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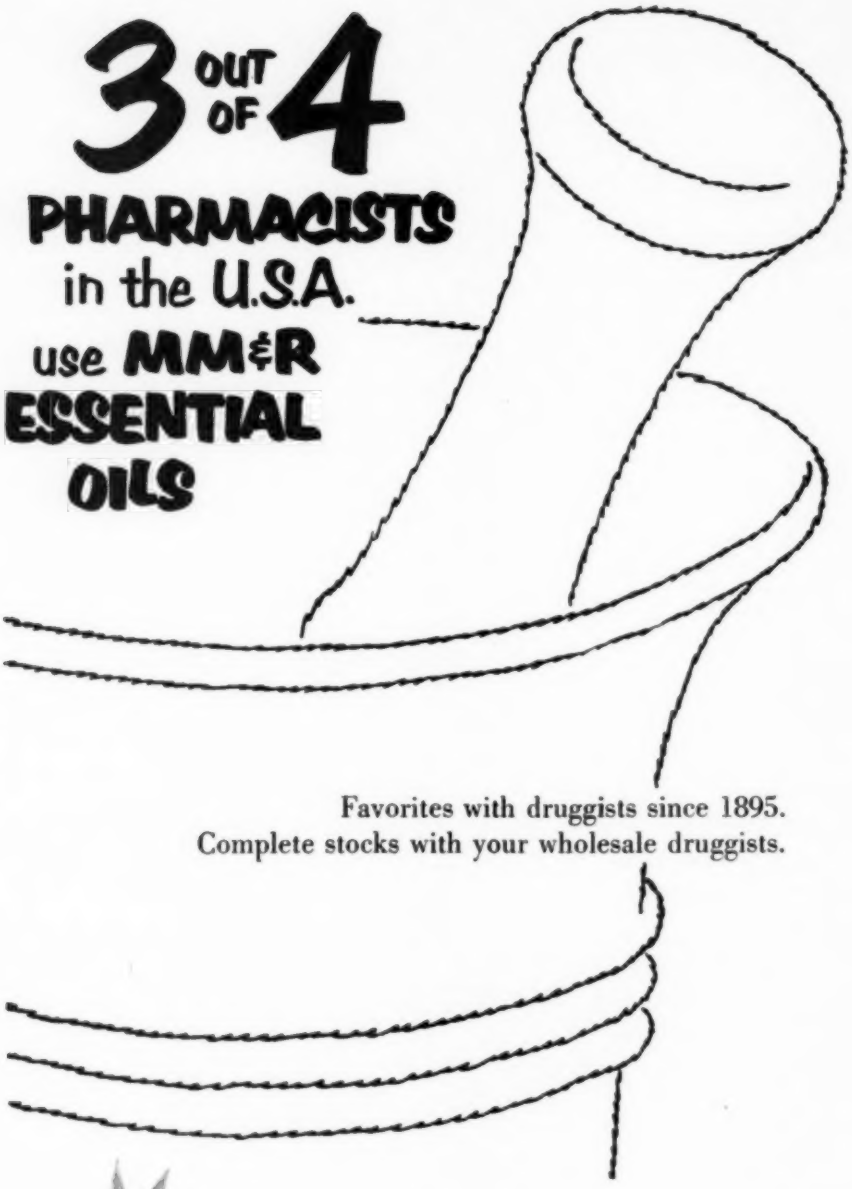

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VOLUME XLI — *MAY, 1952* — NUMBER 5 — CONSECUTIVE NO. 10

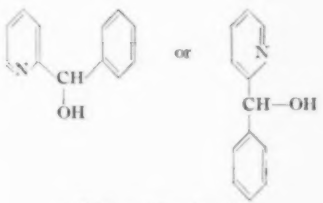
The Synthesis of Some Substituted Pyridine and Piperidine Compounds from Dipicolinic Acid*,†

By MYRON R. BUCHDAHL‡ and TAITO O. SOINE§

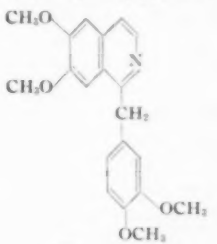
A general procedure for the synthesis of pyridine carbinols by the condensation of dipicolinic acid with aldehydes and ketones and their reduction to the corresponding piperidine and N-methylpiperidine derivatives has been described.

SPERBER and co-workers at Schering Laboratories, while making a series of pyridyl-substituted alkamine ethers in 1949 (1), synthesized α-hydroxybenzylpyridine along with several other intermediate compounds. This type of compound appeared to be well worth investigating for two reasons:

(*a*) Compounds of the α-hydroxybenzylpyridine type structure would warrant study from the standpoint of possible antispasmodic activity because the structure has a formal although distant similarity to that of papaverine. Inasmuch as tetrahydropapaverine retains significant antispasmodic activity, the reduction of the pyridine ring should give potential antispasmodics as well. This has been partially confirmed by Winterfeld (2), who showed that α-(N-methylpiperidyl)-methylpropylcarbinol has papaverine-like action on the muscles of the intestine.



α-Hydroxybenzylpyridine



Papaverine

(*b*) Reduction of the pyridyl nucleus to the corresponding piperidine nucleus gives structures that have a similarity to ephedrine. Marsh (3) has pointed out that increasing the molecular weight of the N-substituent in the potent pressor substance, arterenol, has a marked effect toward lessening the pressor action. In a series of N-alkyl arterenols he found that, in the case of the secondary and tertiary butyl derivatives, the pressor effect is absent but a depressor effect is

* Received November 5, 1951, from the University of Minnesota, Minneapolis.

† Abstracted from a thesis presented to the Graduate Faculty of the University of Minnesota by Myron R. Buchdahl in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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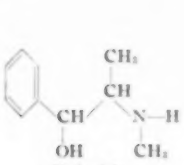
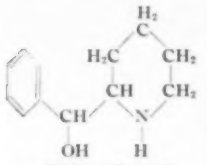
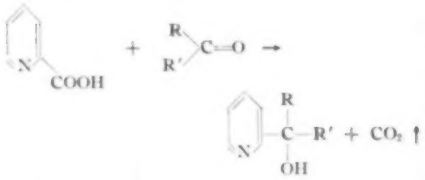

Ephedrine


α-Hydroxybenzylpiperidine

quite prominent. On this basis it would be reasonable to expect the reduced compounds to have a lessened pressor effect and quite possibly a depressor effect. The preliminary pharmacological testing has borne out this prediction.

From a survey of the literature, the most favorable approach to the problem was to synthesize such pyridine carbinols by a procedure that had been utilized not only by Sperber (1) but also by Dyson and Hammick (4). This method consisted of refluxing an α-pyridine carboxylic acid with an aldehyde or ketone for which the refluxing temperature was somewhat higher than the decarboxylating temperature of the pyridine acid. Although the yields are low, the ease of operation and isolation of the carbinol renders the method a useful procedure.



Dipicolinic acid was deemed preferable to α-picolinic acid for the following reasons:

(*a*) The products from α-picolinic acid are identical with those prepared from dipicolinic acid. This was shown by condensing propiophenone with each acid and comparing the products by mixed melting points and also by analysis. The only exception encountered was the dicarbinol obtained from the condensation of benzaldehyde with dipicolinic acid. This atypical compound had previously been prepared by Soine and Andersson (5) from the reduction of 2,6-dibenzoylpyridine with platinum oxide at low temperatures and pressure. The latter, however, erroneously reported the compound to be 2,6-dibenzoylpiperidine. This discrepancy was shown beyond all doubt when a mixed melting point taken of the dicarbinol condensation compound and the original sample of supposed 2,6-dibenzoylpiperidine showed no depression.

(*b*) At the outset of the research it seemed possible that aldehydes other than benzaldehyde might be found to yield bilateral substitution products. These would be of interest in permitting a comparison of mono- and di-substituted compounds.

(*c*) Dipicolinic acid is easily prepared by the permanganate oxidation of commercially obtainable 2,6-lutidine (6). This oxidation is as easy to perform as the oxidation of α-picoline to α-picolinic acid by permanganate and permits the isolation of the free acid in contrast to α-picolinic acid which is isolated as the hydrochloride.

(*d*) On the basis of the yields obtained from condensing dipicolinic acid with propiophenone and α-picolinic acid with propiophenone, it seemed probable that dipicolinic acid would give better yields of monosubstituted products than α-picolinic acid. This would be a consequence of the bilateral nature of dipicolinic acid which offers two α-positions for the decarboxylation reaction instead of the single position in α-picolinic acid.

Ashworth and Hammick (7) had suggested that the mechanism of the condensation reaction is similar to the cyanohydrin addition reaction for aldehydes and ketones. It is also feasible to conceive of the formation of a "Zwitterion" by the free acid and subsequent addition to the aldehyde or ketone as follows:

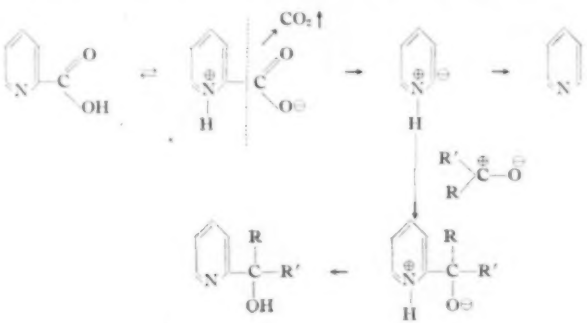


TABLE I.—PYRIDINE DERIVATIVES PREPARED BY THE CONDENSATION OF DIPICOLINIC ACID WITH ALDEHYDES AND KETONES

R	R′	Molecular Formula of Free Base	Parent Aldehyde or Ketone	Yield Obtained, %	M. P. Free Base, °C.	M. P. HCl, °C.	Calculated C	Calculated H	Found C	Found H
—H	—C_6H_5	$C_{19}H_{17}NO_2$[a, e]	Benzaldehyde	30.0	125	179	69.5	5.5	69.6	5.7
—H	—C_6H_5	$C_{12}H_{11}NO$[b, c]	Benzaldehyde	23.3	..	78	Known compound, see references (1) and (4)			
—CH_3	—C_6H_5	$C_{13}H_{13}NO$[d, e]	Acetophenone	38.4	..	189	66.2	6.0	66.6	6.3
—C_2H_5	—C_6H_5	$C_{14}H_{15}NO$	Propiophenone	38.6	87	..	78.8	7.1	78.9	7.1
—C_2H_5	—C_6H_5	$C_{14}H_{15}NO$[b]	Propiophenone	23.3	87	..	Mixed m. p. with above			
—C_3H_7	—C_6H_5	$C_{15}H_{17}NO$	Butyrophenone	19.0	84	..	79.3	7.5	79.0	7.9
—C_4H_9	—C_6H_5	.....	Valerophenone	..	..	..	..	..	..	..
—CH_3	—C_6H_4—CH_3 (4)	$C_{14}H_{15}NO$[c]	Methyl-*p*-tolyl ketone	15.6	..	159	67.3	6.5	67.7	6.7
—C_6H_5	—C_6H_4—CH_3 (4)	$C_{19}H_{17}NO$	Phenyl-*p*-tolyl ketone	3.0	105	..	82.9	6.2	82.3	6.5
—C_6H_5	—C_6H_5	$C_{18}H_{15}NO$[c]	Benzophenone	13.0	107	168	82.7	5.8	82.2	6.1
—H	—$C_6H_4(OCH_3)$ (4)	$C_{13}H_{13}NO_2$[d, e]	Anisaldehyde	21.0	95	154	62.0	5.6	62.4	5.2
—CH_3	—$C_6H_4(OCH_3)$ (4)	$C_{14}H_{15}NO_2$[c]	*p*-Methoxy acetophenone	10.6	..	169	63.3	6.1	63.3	6.2
—H	—$C_6H_3(OCH_3)_2$ (3, 4)	$C_{14}H_{15}NO_3$[d]	Veratric aldehyde	1.8	95	185	68.5	6.2	68.0	5.6
—H	—$C_6H_3(Cl)_2$ (3, 4)	$C_{12}H_9Cl_2NO$[c]	3,4-Dichloro benzaldehyde	14.7	..	201	49.6	3.5	50.1	3.9
—H	—$C_6H_3(OCH_3)_2$ (2, 3)	$C_{14}H_{15}NO_4$[c]	2,3-Dimethoxy benzaldehyde	5.8	..	170	59.7	5.7	59.4	5.9
—H	—$C_6H_3(OH)_2$ (2, 4)	.....	2,4-Dihydroxy benzaldehyde	..	..	..	..	..	..	Decomposition in original mixture

[a] The only dicarbinol formed from dipicolinic acid.
[b] These reactions employed α-picolinic acid.
[c] Reported by Sperber with yields and melting points (1).
[d] Reported by Sperber as oils (1).
[e] The calculated and experimental analytical values are for the hydrochloride salt.

During the preparation of the compounds found in Table I, over 40 reactions were attempted, and as a result the following generalizations can be made:

(*a*) The greater the ratio of aldehyde or ketone to diacid the better chance there is for the condensation to occur.

(*b*) Heating at temperatures below the boiling point of the reaction mixture but still sufficient to cause decarboxylation enhanced the yields somewhat.

(*c*) The use of an impure aldehyde or ketone resulted in a decreased yield of product.

(*d*) The properties of the carbinols vary somewhat and in order to obtain optimum conditions, each reaction should be studied as a separate entity.

Those pyridine compounds that could be converted to water-soluble salts have been tested by Dr. Wallace White of this college for antispasmodic activity. Preliminary pharmacological study has shown these compounds to possess antispasmodic activity in a varying degree. The results of this testing will be published by Dr. White in a later paper.

The compounds that are found in Table II were prepared by the catalytic hydrogenation of the corresponding carbinol of pyridine with platinum oxide in an acidic alcoholic medium. The yields obtained were very low, possibly due to cleavage of the pyridine ring under the conditions of the reaction. A single form was isolated in all cases with one exception. These compounds have been tested for their circulatory activity, and preliminary pharmacological study indicates that these monosubstituted compounds are more active than the two forms of 2,6-di-(α-hydroxybenzyl)-piperidine which were prepared by the catalytic reduction of the corresponding dicarbinol. These latter compounds have previously been reported by Soine and Andersson (5) and later by Jenkins and Bachmann (8). All of the monosubstituted compounds exhibited a depressor effect. In the near future, these compounds will also be tested for antispasmodic action, and a full report of the pharmacological activity will be made by others.

It seemed advisable to prepare and test some of the N-methylpiperidine derivatives as well as the piperidine compounds. Because of the small amounts of reduced forms available, it was not practical to prepare the N-methyl forms from the piperidine homologs. As a result, the unreduced pyridine carbinols were converted to quaternary salts by refluxing with dimethyl sulfate in ether. The quaternary salts were then reduced in the same manner employed for the piperidine compounds. The compounds found in Table III were prepared in this manner. These compounds have been tested for circulatory activity, and preliminary pharmacological findings show them to be more toxic and possibly more active than are the piperidine compounds. The action was depressor in nature. As in the case of the piperidine compounds, the N-methyl derivatives will also be tested for antispasmodic action and reported on later by others.

EXPERIMENTAL

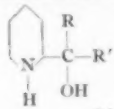
The Condensation Reaction.—The pyridine derivatives described in Table I may be prepared in the following manner. A ratio of 20 moles of aldehyde or ketone to 1 mole of dipicolinic acid is placed in an ordinary reflux setup and refluxed for a period of thirty hours or until carbon dioxide ceases to be evolved. Upon cooling, 300 cc. of ether is added to the reaction mixture. This ethereal extract is then shaken with 5% (w/v) hydrochloric acid until the extractions fail to give a cloudiness with sodium carbonate. The combined extractions are washed with three generous portions of ether and warmed on a steam bath to an ether-free condition. The aqueous solution is brought to a *p*H of about 8 by carefully neutralizing with powdered sodium carbonate. The crude base is extracted from the alkalinized mixture by shaking with ether. The ethereal extractions are washed with water and dried over anhydrous sodium sulfate. If the free base is to be isolated, it is obtained by evaporating the ether from the ethereal extract and then crystallizing the residue from diluted alcohol. To obtain the hydrochlorides, a dried ethereal solution of the free base is treated with a slight excess of ethereal hydrogen chloride. The resulting precipitate of the base hydrochloride is recrystallized from a mixture of absolute alcohol and ether. In a few cases it was found impossible to prepare a crystalline free base, and in some cases a crystalline hydrochloride could not be prepared. Only in the case of the reaction product of dipicolinic acid with valerophenone and 2,4-dihydroxybenzaldehyde, respectively, was it impossible to obtain either the free base or hydrochloride in a crystalline state. The compounds prepared are described in Table I.

The Catalytic Reduction of α-Pyridine Carbinols.—A mixture of the material to be reduced, 200 cc. of absolute alcohol (slightly acidified with alcoholic hydrogen chloride) and 0.25 Gm. of platinum oxide per 5 Gm. of material to be reduced was introduced into a ½-L. pressure bottle.

The reductions were carried out in a Parr low-pressure hydrogenation apparatus at an initial pressure of 45 p. s. i. of hydrogen. Upon completion of the hydrogenation, the catalyst was removed by filtration. By alternately chilling and concentrating, the various reduction products were obtained by fractional crystallization. Each fraction was then recrystallized several times from absolute alcohol and absolute ether. The compounds prepared are described in Table II.

The Preparation and Hydrogenation of Quaternary Salts.—Quaternary salts were prepared by refluxing the free bases of pyridine carbinols with an

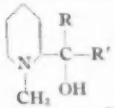
TABLE II.—HYDROCHLORIDES OF PIPERIDINE CARBINOL DERIVATIVES



R	R′	Molecular Formula	Compound Reduced (Pyridine Carbinol), Gm.	HCl Obtained (Piperidine Carbinol), Gm.	M. P. HCl, °C.	Calculated Cl Anal.	Actual Cl Analysis
—CH_3	—C_6H_5	$C_{12}H_{19}NO\cdot HCl$	7.2[a]	1.65	199	14.67	14.56
—C_2H_5	—C_6H_5	$C_{14}H_{21}NO\cdot HCl$	7.0[b]	1.9	254	13.86	13.95
—C_3H_7	—C_6H_5	$C_{15}H_{23}NO\cdot HCl$	2.5[b]	1.3	211	13.14	12.94
—C_6H_9	—C_6H_5	$C_{16}H_{25}NO\cdot HCl$	9.0[c]	3.6	206	12.49	12.76
—H	—C_6H_4—OCH_3 (4)	$C_{13}H_{19}NO_2\cdot HCl$	3.5[b]	0.8	216	13.75	13.53
—H	—C_6H_3—$(Cl)_2$ (3, 4)	$C_{12}H_{18}Cl_2NO\cdot HCl$	5.0[a]	0.6	244	11.95	11.99
—CH_3	—C_6H_4—CH_3 (4)	$C_{14}H_{21}NO\cdot HCl$	3.5[a]	1.0	218	13.87	13.80
—CH_3	—C_6H_4—OCH_3 (4)	$C_{14}H_{21}NO_2\cdot HCl$	5.5[c]	0.6	209	13.04	13.28

[a] Crystalline hydrochloride.
[b] Crystalline free base.
[c] Oily free base.

TABLE III.—HYDROCHLORIDES OF *N*-METHYLPIPERIDINE CARBINOL DERIVATIVES



R	R′	Molecular Formula	Pyridine Carbinol Used, Gm.	*N*-Methyl HCl, Gm.	M. P. *N*-Methyl HCl	Calculated Cl Anal.	Actual Cl Analysis
—H	—C_6H_5	$C_{20}H_{25}NO_2\cdot HCl$[a]	5.0	0.8	220	10.20	9.95
				0.3	244	10.20	10.26
—CH_3	—C_6H_5	$C_{14}H_{21}NO\cdot HCl$	6.0	0.7	177	13.86	14.14
—C_2H_5	—C_6H_5	$C_{15}H_{23}NO\cdot HCl$	4.5	0.5	183	13.14	13.31
—H	—$C_6H_4(OCH_3)$ (4)	$C_{14}H_{21}NO_2\cdot HCl$	4.0	0.6	196	13.04	13.25
—CH_3	—C_6H_4—CH_3 (4)	$C_{15}H_{23}NO\cdot HCl$	4.0	0.6	226	13.14	13.13

[a] This compound is a symmetrical disubstituted dicarbinol.

excess of dimethyl sulfate in an ether medium on a steam bath for one hour. Upon chilling, the quaternary salt separated from the ethereal solution as an oil. The oil was washed several times with anhydrous ether until free of dimethyl sulfate. The oils were reduced in exactly the same manner as were the pyridine compounds.

The reduction mixture, upon completion of hydrogenation, was stripped of its solvent. The residual oil was dissolved in water and neutralized with sodium carbonate. The N-methylpiperidine free base was extracted with ether and the ethereal solution was dried over anhydrous sodium sulfate. A slight excess over the theoretical amount of ethereal hydrogen chloride was added to the ethereal solution and the N-methylpiperidine hydrochloride separated as an oil. The resulting oils were crystallized from a mixture of acetone and ether. Once crystallized, these compounds were easily recrystallized from absolute alcohol and absolute ether. The compounds prepared are described in Table III.

Attempts to reduce the methiodide of di-(α-hydroxybenzyl)pyridine resulted in failure.

SUMMARY

1. A series of 11 new pyridine carbinols was prepared by the condensation of dipicolinic acid with an aldehyde or a ketone. These compounds have been subjected by others to a preliminary pharmacological study and have exhibited antispasmodic activity.

2. Eight new piperidine monocarbinols were prepared by the catalytic hydrogenation of the corresponding pyridine monocarbinols. Preliminary pharmacological investigations showed these compounds to have a definite circulatory effect. In the near future they will be tested for antispasmodic activity.

3. Five new N-methylpiperidine compounds were prepared by the catalytic hydrogenation of the corresponding quaternary pyridine methosulfates. These compounds have been shown to exert a circulatory effect that is similar to that exhibited by the piperidine homologs. The N-methyl compounds, however, appear to be more toxic. These compounds will also be tested for antispasmodic action and will be reported on by others.

REFERENCES

(1) Sperber, N., *J. Am. Chem. Soc.*, **71**, 886(1949).
(2) Winterfeld, K., *Arch. Pharm.*, **273**, 315(1935).
(3) Marsh, D., *J. Pharmacol. Exptl. Therap.*, **93**, 114 (1948).
(4) Dyson, P., and Hammick, D., *J. Chem. Soc.*, **1937**, p. 1724.
(5) Soine, T., and Andersson, N., THIS JOURNAL, **39**, 463 (1950).
(6) Soine, T., and Buchdahl, M., *ibid.*, **39**, 421(1950).
(7) Ashworth, M., and Hammick, D., *J. Chem. Soc.*, **1939**, p. 809.
(8) Jenkins, G., and Bachmann, R., THIS JOURNAL, **40**, 46(1951).

A Preliminary Phytochemical Study of *Tillandsia usneoides* L. (Spanish Moss)*

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A phytochemical study of *Tillandsia usneoides* L. (Spanish moss) was made. A flavonol type of glycoside having weak antibacterial (*M. aureus*) action was isolated. Attempts to obtain it in crystalline form were unsuccessful. Chromatographic separation by means of activated alumina proved to be a satisfactory method for the processing of large quantities of plant material. The presence of the glycoside appeared to vary greatly with changes in seasons and humidity.

INVESTIGATIONS of Spanish moss have been largely limited to its application in the upholstery industry or to ash analyses. Schorger (1) made a carbohydrate analysis of Spanish moss in 1927. He reports a methyl-phenol-ether-glucoside, thought by us to be of a flavonolic nature. The inorganic analyses of Spanish moss were begun as early as 1865 by De Luca (2). Others making ash and elemental analyses include Pickell (3) in 1890, Halligan (4) in 1909, and Wherry and Buchanan (5) in 1926. The results of their work vary considerably according to the location from which the moss was obtained. Elements reported and the ranges of their concentration include sodium calculated as sodium oxide 2.7 to 25 per cent of the ash, potassium oxide 6–25 per cent, calcium oxide 3–12 per cent; also present are magnesium, iron, aluminum, phosphorus, and chlorine. The water content of the green moss ranges from 60 to 70 per cent during the summer months. The protein content (14 per cent) was also reported by Pickell (3). In 1904 Billings (6) reported the growing habits, ability of the Spanish moss to withstand drought, and a histological study of the various plant parts.

A preliminary report by Weld (7) in 1945 stated that alcoholic extracts of Spanish moss possessed antibiotic activity. The latest publication concerning Spanish moss was a report of the ascorbic acid and carotene content by French and Abbott (8).

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The initial steps in this investigation consisted of numerous small extractions (50–100 Gm.) to determine the optimum conditions and solvents for a large extraction. These extracts were routinely tested against *Micrococcus pyrogenes* var. *aureus* for germicidal activity using the twenty-four-hour F. D. A. agar cup method. During this interval it was found that the germicidal activity varied with the season, occurring most abundantly during the summer months and being absent in extractions made between mid-December and early May. No germicidal activity was found in extracts made from dried or dead moss.

Methanol, ethanol, and acetone appeared to be equally effective as extracting solvents, but acetone was used because of its more selective action, and the ease of its removal by extraction with petroleum ether and chloroform in the process of removing other interfering extracted substances. The use of chromatography with a column of activated alumina served to further purify and concentrate the extract.

Chromatography of the wash solutions (petroleum ether and chloroform fractions) and their constituents indicate that Spanish moss produces a number of substances, several of which are worthy of further study in addition to completion of the work begun here.

The tough inner hair of the Spanish moss presented a special grinding problem in that the long strands quickly clogged any worm-gear type grinder. This problem was solved by the use of a tractor-powered hammer mill. The particle size was not as fine by this method as was obtained with the Waring blendor; therefore, the acetone extraction was followed by a water extraction and processing of the aqueous extract by the lead method. The processing of the acetone extract and chromatography of the larger volume followed the results obtained in the earlier studies with 50–100 Gm. samples.

Studies of the chemical nature and determination of the physical properties of the antibiotic principle have not been as specific as desired since the extreme hygroscopic nature of the substance and the inability to obtain a pure crystalline product have been major obstacles in this work. General characteristics of the ma-

terial indicate a difficultly hydrolyzable glycoside of a flavonol-type compound.

EXPERIMENTAL

Choice of the Method and Solvent.—Extractions were made on 50–100 Gm. samples of Spanish moss to determine the best solvent for a selective extraction. Special attention was directed toward the isolation of the antibiotic principle reported by Weld (7). Therefore, samples of the Spanish moss were tested against *Micrococcus pyrogenes* var. *aureus* (*Staphylococcus aureus*) using the twenty-four-hour F. D. A. agar cup method, 0.2-cc. sample per cup.

It was found that comminution with the Waring blendor followed by maceration for six to twelve hours gave the best zones of inhibition with little difference in inhibition being shown with extracts of methanol, ethanol, and acetone. Chloroform, benzene, ether, and petroleum ether extracts showed no bactericidal activity. Iron, zinc, and copper filings had no effect and it was concluded that the type of maceration vessel for a large extraction was immaterial. The extracts from each of the above determinations showed a 1.5 to 2.0 mm. zone of inhibition on samples in which the volume was adjusted to 1 cc. of extract, equivalent to 1 Gm. of the fresh moss. A solvent blank was run for each sample.

It was found that by extracting the above extracts with petroleum ether (b. p. 40–60°) an immiscible system was formed with each of the three solvents (ethanol, methanol, acetone), and that chlorophyll and other pigments could be effectively removed. Gilbert (9) suggested this technique particularly in reference to the acetone extract. Chloroform was also found to form an immiscible system with the original acetone extract due to the high water content of the moss extract after the petroleum ether treatment.

Acetone was accordingly chosen as the solvent. The acetone extract from the moss was a clear green solution heavily laden with chlorophyll. After shaking out with petroleum ether, the volume of the acetone extract was reduced approximately 30%. At this point the color was yellow to yellowish green. On shaking out with chloroform, a much larger portion of the acetone was removed so that the aqueous portion was reduced to 10–30% of the original volume according to the water content of the original moss. The color of the aqueous acetone portion was a clear red. This color change is thought to be due at least in part to the acid content of the chloroform since the acidity was increased during this step, and on neutralization the color reverted to yellow. The extract showed a stronger bactericidal activity than the earlier tests, a 0.2-cc. sample showed a 1.0 to 1.5 cm. inhibition zone. The chloroform-acetone portion occasionally showed slight activity, but this loss was considered negligible in view of the function of chloroform in removing wax and remaining portions of the pigments. Germicidal tests of the petroleum ether fraction of the acetone extract were invariably negative.

Treatment of the aqueous acetone extract after successive extraction with petroleum ether and chloroform with a 10% lead acetate solution yielded a copious green precipitate which could only be filtered with the aid of a filter agent (Hyflo Supercel, Johns-Manville). The filtrate was treated with hydrogen sulfide, and both portions were tested for antibiotic activity. No inhibition was found.

The use of chromatography was next investigated as a means of purification. The simple fast technique of disk chromatography described by Williams (10) was used for the testing of adsorbents and elutriants.

Using this method with the aqueous acetone extract, the most satisfactory adsorbent was found to be activated alumina (Grade F-20, 200 mesh, Aluminum Company of America). Calcium hydroxide, magnesium carbonate, and magnesium oxide reacted chemically with the acidic portion of the extract. Elution attempts were unsuccessful with fuller's earth.

Because of the limited volume of the disk chromatograms, studies were continued, using activated alumina in small columns of 10 mm. diameter and 25 cm. long. Each fraction of the acetone extract was chromatographed and the following results obtained:

Petroleum Ether Fraction.—Five bands, beginning with the uppermost or most strongly adsorbed: light green, dark green, light green, yellow, and orange. The latter two were very weakly adsorbed and followed the solvent front quite closely.

Chloroform Fraction.—Three bands, beginning with the uppermost: a deep yellow, a fluorescent yellow which was destroyed by direct light, and light yellow.

Aqueous Acetone Fraction.—Two bands: the uppermost band was an olive green which blended into a yellowish-orange band immediately below. As shown below, on elution with acidic methanol the green portion remained adsorbed in a broad band and the lower band was gathered to form a bright orange band which moved uniformly downward upon elution.

The flowing chromatogram technique was found to be the most satisfactory in the purification of the aqueous acetone portion in that the orange band was found to possess the antibiotic activity. A sample (0.2 cc.) on neutralization and tested against *M. aureus* showed only a 2.5 to 3.0 cm. zone of inhibition by the agar cup method. The final extract in this study was concentrated approximately forty times that of the original acetone extract.

After adsorption of the aqueous acetone extract, acetone was added and used to wash the unadsorbed extract on through the column. It also served to remove water from the alumina so that band formation took place on elution. Five per cent acetic acid in methanol was found to be the best combination for elution of the orange band. The extraction scheme shown in the Extraction Flow Sheet thus completed the work done on the choice of the method and solvents to be used in a large-scale extraction of the Spanish moss.

Large-Scale Extraction.—The Spanish moss was gathered in two portions a few days apart during the week of August 3, 1950, within a few miles west of Gainesville. It was sorted by hand to remove leaves, twigs, and old dead moss. It was then ground with a hammer mill into fragments approximately one-fourth inch in length. A total of 116.5 pounds was obtained (Lot A).

Lot A was macerated with a total of 70 gallons of acetone in two steps for forty-eight hours. A total volume of 56.5 gallons (Step I) was obtained.

It appeared that extraction was incomplete although two macerations with acetone had been made. Therefore, tap water was added to the residue of lot A immediately after the second acetone fraction had been drained off, and it was allowed to macerate again for forty-eight hours. The water extract was drained off. Expression of the marc yielded a total aqueous extract of 24.5 gallons (Step Ia).

A small sample of the combined acetone extracts was evaporated to dryness and the mass placed in a desiccator overnight; yield, 0.652 Gm. per 100 cc. or 2.64%. A later acetone extraction using more finely ground moss yielded 3.95% total extract. The weight and per cent solids were not calculated for the aqueous extract at this stage of the extraction process.

Purification of the Acetone Extract (Step I).—The acetone extract was treated in 3-gallon lots. Each portion was placed in a 5-gallon carboy and a gallon of petroleum ether was added. After thorough shaking and standing a few minutes, the mixture separated into two layers. A siphon system was then inserted and the lower acetone layer was removed. This first petroleum ether fraction contained a large portion of the chlorophyll and other pigments. However, a second treatment with one-half gallon of petroleum ether was performed, thus assuring a more complete removal of these substances and further reduction of the acetone content of the original extract (Step II).

After siphoning off the acetone from the second petroleum ether treatment, the water content was high enough to form an immiscible system with chloroform. The shake-out procedure was the same as given above. One gallon of chloroform was used in a single extraction. The second shake-out with chloroform was omitted because of emulsification, and the further reduction of the volume of the acetone extract did not warrant it.

The above treatment served to reduce the volume of the acetone extract from 56.5 gallons to 11 gallons. The water content of this concentrate was approximately 80% (Step III). Therefore, it was stored in the refrigerator at 10° until processed in the alumina column.

The adsorption column assembly consisted of a 5-gallon carboy as the receiver for unadsorbed filtrate, a No. 2 Büchner funnel lined with glass wool, and $3^1/_2$ in. x 4 ft. glass tube. The stopper of the funnel was vented to allow air to escape.

The column was prepared by adding the activated alumina through a funnel so that the powder flow was directed to the center of the column at all times. Five pounds of alumina was used for each lot, and it was added in one portion, that is, the adsorbent was not added in small portions with tamping or suction to settle it and to fill voids between additions of the material. It was found that, when added in a single portion, the activated alumina, grade F-20, settled in such a way that the coarser particles were in the lower portion and the finer material on top. The voids could then be filled uniformly by gentle tapping at the base of the column.

The column was then ready to be used. Two liters of the aqueous acetone extract filled the reservoir above the alumina. The beginning flow was rapid, but as hydration of the column increased, the velocity decreased. This hydration of the column was accompanied by considerable heat. The first portions of the filtrate were colorless, but, after 1 to 1.5 L. had passed through, were light amber. The column capacity was found to be sufficient for 8 L. of the concentrated extract (Step IV).

The next step in the process consisted of a 2-L. methanol wash. This was added to the column just as the last of the extract flowed into the alumina. This wash procedure had two functions: (*a*) it carried through the unabsorbed extract; (*b*) it removed enough water from the hydrated alumina so that, on developing and eluting, a sharp band could be obtained (Step V). The acetone was colorless when it left the column.

The arrangement of the bands and their respective colors was the same as described above using the small columns.

As the last portion of the acetone was disappearing into the alumina, 5% acetic acid in methanol was added. The receiver was changed at this time in preparation for the collection of the concentrated elute. The orange band began to form and move downward. Elution of the orange band required 3 L. of the 5% acetic acid-methanol mixture (see Fig. 1). Several liters of methanol were added finally to wash through any material which could now be eluted and the last portions of the acetic acid-methanol elute (Step VI).

Adsorption and elution for this study were now considered complete. A total of five such columns were required to chromatograph the total volume of aqueous acetone extract. Attempts to elute further with methanolic hydrochloric acid were unsuccessful.

The methanol-acetic acid concentrate from the five columns was combined, and made a volume of approximately 15 L. so that a second concentration from 11 gallons had been achieved without the aid of heat.

The above concentrate was reduced to a syrupy consistency by distillation under reduced pressure (185–200 mm. Hg) and a temperature not exceeding 40°. The volume had now been reduced to approximately 600 cc. (Step VII). This syrupy mass was placed in a desiccator over potassium hydroxide and was stored in a refrigerator at 10°. One hundred and fifteen grams of crystalline sodium acetate separated from the syrup. This salt was a by-product of the sodium salt impurities occurring in the alumina and sodium ions from the moss extract. Sodium, calculated as sodium oxide, has been reported (3) to occur as high as 25% of the ash of the dried Spanish moss. The sodium acetate isolated here was identified by ignition of the salt, and preparation of the silver acetate followed by ignition and weighing. The gummy residue of the alumina concentrate, after removal of most of the water and acetic acid under reduced pressure evaporation and desiccation, weighed 140 Gm. There was still some sodium acetate present.

Purification of the Water Extract (Step Ia).—As mentioned earlier, the water extract containing some residual acetone was made because it appeared that extraction by the acetone was incomplete. This aqueous extract was processed by the lead method as outlined by Rosenthaler (11) with modifications suggested by Sokoloff (12).

The aqueous extract (24.5 gallons) was divided into three portions and 4 L. of 30% lead acetate

EXTRACTION FLOW SHEET

Step I
Fresh green moss, finely ground and macerated 48 hours in acetone, drained, and marc expressed*
Step II
Acetone extract containing 20–30% water extracted with petroleum ether*
Petroleum ether fraction containing chlorophyll and other pigments**
Step III
Acetone extract containing 30–40% water extracted with chloroform*
Chloroform fraction containing wax, remaining pigments and fluorescent compound***
Step IV
Acetone extract containing 80–90% water adsorbed on Alumina F-20*
Filtrate first colorless then light amber**
Step V
Column washed with methanol to remove unadsorbed extract and dehydrate*
Washings light green then colorless**
Step VI
Column eluted with 5% acetic acid in methanol*
Column has a strong green color at top which fades to almost white at bottom**
Step VII
Methanol-acetic acid concentrate, dark amber*

* Shows bactericidal activity.
** Shows no bactericidal activity.
*** Shows occasional slight bactericidal activity.

solution was added to each portion. A copious green precipitate formed and was allowed to settle overnight. The clear amber supernatant liquid was siphoned off, and the lead precipitates were combined for further settling. The supernatant liquid was made alkaline to litmus (*p*H 8.2) with concentrated ammonium hydroxide. A bright yellow precipitate formed. This precipitate was much finer and did not settle as readily as the first. After settling overnight the supernatant liquid was siphoned off, and the basic lead precipitate was placed in 1-L. Imhoff cones to concentrate further. After settling six to twelve hours the supernatant liquid was decanted, and Hyflo Supercel was added and the remainder of the liquid removed by suction filtration. The filter cake was washed three times with a 1% ammonium hydroxide solution, and then stored under methanol until the entire lot had been processed to this stage.

The basic lead complex was decomposed by dispersion of the filter cake in methanol and the addition of 8.5% phosphoric acid in methanol until the mixture was acid to Congo red paper (*p*H 3). The lead phosphate was removed by suction filtration, and the filtrate was neutralized to *p*H 5 with concentrated ammonium hydroxide. After chilling and filtering to remove ammonium phosphate, the methanolic solution was concentrated under reduced pressure distillation at a temperature not exceeding 40°. After removal of the methanol and a large portion of the water, there remained a gummy residue (144 Gm.) equivalent to 0.21% of the Spanish moss extracted. Bactericidal action was shown by this concentrate, but it was not as strong as that shown by the samples isolated by the column method.

Chemical Nature of the Antibiotic Substance.—The residue obtained from concentration of the alumina column elute and still contaminated with a slight amount of sodium acetate was orange-brown in color, very hygroscopic, and foamed readily upon solution in water. Elemental analyses by the sodium fusion method were made for halogens, sulfur, and nitrogen. Negative results were found

for each. Phosphorus was tested for and found absent.

Extensive tests were made for the possibility that the substance was of a saponin nature. It was found to emulsify mercury readily and to foam excessively on shaking. However, other standard tests were found negative. The compound did not hemolyze the red blood cells of a rabbit, nor did it form a crystalline complex with cholesterol. Digitonin (0.1%) in absolute ethanol failed to give a precipitate. Color reactions performed and found negative included the Liebermann test for phytosterols, the Zimmermann reaction, and diazotization with *p*-nitroaniline.

The method of Jacobs and Simpson (13) for steroidal saponins was used in attempting to hydrolyze the substance. Ten grams of the material was dissolved in 50 cc. of 2 *N* ethanolic hydrochloric acid. The mixture was refluxed for twenty hours, and after cooling, ether was added to precipitate sugars and unhydrolyzed material. The solution was then filtered and the filtrate heated on a steam bath to remove the ether and most of the alcohol. It was then extracted three times with toluene and the combined toluene extracts were shaken out with 5% sodium carbonate solution, an amber solution being drawn off each time. Norit was then added to the toluene extract and heated to boiling. It was then filtered while hot. The toluene filtrate was a light amber color. After drying over calcium carbonate and evaporation on the steam bath, a soft wax-like residue remained, 0.2 Gm. or 2% yield. This material also failed to give the digitonin reaction or the Liebermann color reaction for sterols. Therefore, it was concluded that the sterol reported by Schorger (1) was apparently found only in the wax (under investigation by another member of this college) and not as a steroidal saponin.

Elimination of the sodium acetate from the gummy residue was accomplished by dissolving in ethanol and passing dry hydrogen chloride gas through the solution for several minutes. Since this operation was carried out in a cold solution for a very short time, no esterification seemed likely nor was apparent. Sodium chloride was precipitated immediately and was filtered off. The filtrate was then concentrated by distillation under reduced pressure at a temperature not exceeding 40°. The dark green residue, after complete removal of the ethanol, water, and acetic acid, dried to a varnish-like film. It was also found that the gummy residue could be obtained in powder form by grinding in a mortar under acetone in the presence of dry ice. This procedure, followed by several rapid washes with acetone, filtration, and rapid insertion in a desiccator, gave an amorphous yellowish-green powder which was extremely hygroscopic. The melting point of this powder was found to be 91–93° (uncorr.) on one trial and decomposition started at 180° (uncorr.) using a sample precipitated from hydrated butanol (7% water) by ether and dried under vacuum. This indicates the possibility of a mixture. Several attempts were made to form an acetyl derivative both by the use of pyridine and by sodium acetate methods, but without success.

The reaction of the compound in alkaline and acid solution is that of an indicator-type pigment. It is deep yellow in alkaline solution down to *p*H 5.5 to 6. It then becomes colorless and upon solution in concentrated acid is red, or after being kept for some time, yellow. Other tests made and found positive for a flavonol-type compound were reduction with magnesium, zinc, and hydrochloric acid to give a red solution. It is very stable to acid degradation, reduces Fehling's solution on warming, and gives an orange-brown solution with glacial acetic acid on the addition of concentrated sulfuric solution (the oxonium salt failed to separate). The Wilson (14) boric acid test for the formation of a chelated compound between the 5-hydroxyl group and the carbonyl group was found negative.

Shibata and Kimotsuki (15) made a spectrographic study of a number of flavones and related compounds. From their work they were able to point out several regions of the spectrum where absorption generally occurred, one of the stronger band regions being 270–280 mμ. The strongest absorption region for this compound was found at 265 mμ with a weaker band at 355 mμ.

The method of Baker (16) and used by Haley and Bassin (17) in the hydrolysis of iridin was used on a smaller scale to investigate a possible method of hydrolysis of this compound. One gram of the substance was dissolved in 25 cc. of 60% ethanol and 1 cc. of concentrated sulfuric acid was added. This mixture was heated for five hours at 15 lb. pressure (120°). On cooling, the content of the bottle was poured into 25 cc. of 60% ethanol, boiled with charcoal, and filtered. Twenty cubic centimeters of water was added to the filtrate, boiled, and filtered again. When no crystalline material separated after chilling overnight at 10°, the solution was concentrated on a steam bath to approximately 10 cc. Alcohol was added to the hot amber mixture until a clear solution resulted and a few drops of water induced cloudiness. On setting for forty-eight hours at 10°, tiny crystals separated in too slight a yield to be investigated further.

An alkaline degradation of the unhydrolyzed compound was attempted under the following conditions: 10 Gm. of the compound was mixed with 50 cc. of 30% aqueous potassium hydroxide and refluxed for six hours. After cooling, dilute sulfuric acid was added until the solution was strongly acid (*p*H 1). The acidified solution was then extracted with 25-cc. portions of ether until colorless ether washings were obtained. The ether solution was evaporated on the steam bath and a gummy residue obtained (0.2 Gm.). A green coloration was obtained with 1% alcoholic ferric chloride solution. Addition of 5% sodium carbonate gave a brown precipitate rather than a red color. (Reaction shown by *o*-dihydric phenols.)

The ether-soluble residue was redissolved in ether and extracted with three portions of 5% sodium carbonate, 20 cc. each. The sodium carbonate washings were light amber and seemed to be carrying over portions of the phenol. Acidification of the sodium carbonate solution and extraction with ether, followed by evaporation, yielded a gummy residue similar to the original ether extract. No conclusions can be drawn at present as to the composition of this mixture except that the degradation process was incomplete.

Schorger (1) reported a methyl-phenol-ether-glucoside as present. Attempts to form a glucosozone from the compound here were unsuccessful although the viscid mass from the aqueous portion of the acid

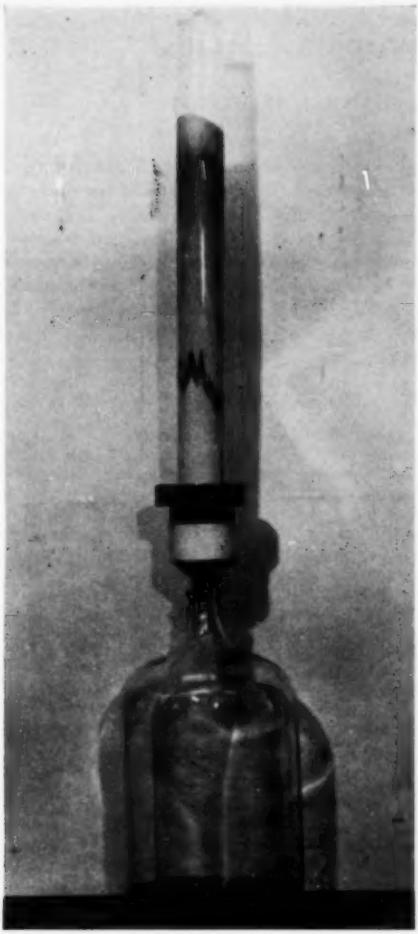


Fig. 1.—Chromatographic column of acetone extract.

hydrolysis was extracted with ethanol and decolorized with Norit, dissolved in water and heated for thirty minutes in the presence of sodium acetate and phenylhydrazine. No precipitation occurred during the heating or on cooling.

Further work on the unsolved portion of this study is under consideration and will be presented at a later date.

SUMMARY

A phytochemical study of *Tillandsia usneoides* L. was made with the isolation and chemical investigation of an antibacterial substance as the primary objectives.

Studies were made to determine the most efficient methods for extraction, purification, and the seasons when bactericidal activity was the greatest. These data were applied in the processing of 116.5 lb. of fresh green moss. Acetone extraction followed by solvent partition with petroleum ether and chloroform yielded a concentrated aqueous acetone extract. This extract concentrate was chromatographed on activated alumina and an orange band eluted by the flow technique with methanol containing 5 per cent acetic acid. Further concentration by reduced pressure evaporation yielded a gummy mass possessing antibacterial activity. Lead precipitation of an aqueous extract was used to supplement the above procedure.

Chemical studies of the noncrystallizable material indicated the possibility that it was a glycoside. Several saponin characteristics were present, but hydrolysis failed to give confirmatory results. Color reactions, solubility, and spectrophotometric data indicate the antibacterial substance to possess many characteristics of a difficultly hydrolyzable flavonol-type glycoside.

REFERENCES

(1) Schorger, A. W., *J. Ind. Eng. Chem.*, **10,** 409 (1927).
(2) De Luca, M. S., *Compt. rend.*, **53,** 244(1861).
(3) Pickell, J. M., Florida Experiment Station Bulletin No. 11, October, 1890.
(4) Halligan, J. E., *J. Ind. Eng. Chem.*, **1,** 206(1909).
(5) Wherry, E. T., and Buchanan, R., *Ecology*, **7,** 303 (1926).
(6) Billings, F. H., *Botan. Gas.*, **38,** 99(1904).
(7) Weld, J. T., *Proc. Soc. Exptl. Biol. Med.*, **59,** 40(1945).
(8) French, R. B., and Abbott, O. D., Florida Agr. Expt. Sta. Tech. Bull., 1948, p. 444.
(9) Gilbert, S. G., unpublished data, U. S. Dept. of Agr. Field Laboratory for Tung Investigation, University of Florida, Gainesville.
(10) Williams, T. I., "An Introduction to Chromatography," Chemical Publishing Company, Inc., Brooklyn, New York, 1947, pp. 37–38.
(11) Rosenthaler, L., "The Chemical Investigation of Plants," translated by S. Ghosh, G. Bell and Sons, Ltd., London, 1930, pp. 31–35.
(12) Sokoloff, B., Bioresearch Laboratory, Florida Southern College, Lakeland, private communication.
(13) Jacobs, W. A., and Simpson, J. C. E., *J. Biol. Chem.*, **105,** 501(1934).
(14) Wilson, C. W., *J. Am. Chem. Soc.*, **61,** 2303(1939).
(15) Shibata, Y., and Kimotsuki, K., *J. Chem. Soc. Japan*, **39,** 771(1918); through Klein, G., "Handbuch der Pflanzenanalyse," Vol. III, Part 2, Julius Springer, Wien, 1932, p. 901.
(16) Baker, W., *J. Chem. Soc.*, **1928,** p. 1022.
(17) Haley, T. J., and Bassin, M., THIS JOURNAL, **40,** 111 (1951).

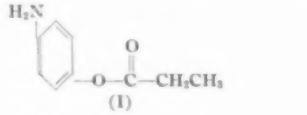
Antispasmodics. I. Phenyl Esters of *Beta*-Dialkylaminopropionic Acids*

By TAITO O. SOINE† and FRANK E. DiGANGI‡

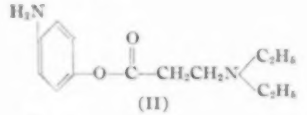
Several *p*-aminophenyl esters of *beta*-dialkylaminopropionic acids [$H_2N—C_6H_4·OOCCH_2CH_2N(R)_2·2HCl$] have been prepared and found to possess moderate antispasmodic activity against both histamine- and acetylcholine-induced smooth muscle spasms.

WHITACRE (1) has pointed out that esters of the type represented by *p*-aminophenyl propionate (I) are not local anesthetics but are instead "fish poisons." Likewise, *p*-aminophenyl-β-diethylaminopropionate (II), which is isomeric with procaine, lacks local anesthetic properties (2). Although lacking in anesthetic properties, the latter compound was noted to have mild antispasmodic properties. This investigation was undertaken to prepare compounds of this general nature to determine whether the antispasmodic activity was significant and whether it could be enhanced. Therefore, compounds of the types indicated by III were prepared with the substituents as indicated.

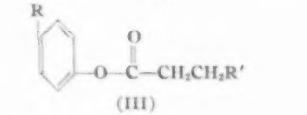
A preliminary testing of the *p*-aminophenyl esters indicated that they did possess antispasmodic activity but not of a highly potent nature. A drawback to their testing, however, was the unstable nature of the compounds in aqueous solution. Therefore, the phenyl and *p*-acetylaminophenyl esters were also made at the request of the pharmacologists testing the products. A full report of the activities of these compounds will be made by others upon completion of the testing.



(I)



(II)



(III)

R	R′
—NO_2	—$N(C_2H_5)_2$
—NH_2	—$N(n\text{-}C_3H_7)_2$
—$NHCOCH_3$	—$N(n\text{-}C_4H_9)_2$
—H	—$N(n\text{-}C_5H_{11})_2$
	—$N(\text{iso-}C_5H_{11})_2$
	—$N(C_5H_{10})$ = (piperidino).

EXPERIMENTAL

***p*-Nitrophenyl-β-chloropropionate.**—To a quantity of 218 cc. of benzene in a tared 1-L. flask, 121.8 Gm. of β-chloropropionyl chloride, 106.5 Gm. of *p*-nitrophenol, and 9.3 Gm. of magnesium turnings were added. The mixture was heated for three hours at 90° on a water bath. The flask was equipped with a reflux condenser. The reaction mixture was then cooled, diluted with ether, and decanted from the magnesium turnings. After the ether and benzene were removed from the decantations, the oily residue was distilled as a heavy yellow oil, b.p. 200° at 17 mm. On cooling, the oil crystallized and was recrystallized from alcohol and water; m.p. 36–37°. The over-all yield for the reaction was 60% of theoretical. In later runs, 100 Gm. of each reactant and 9 Gm. of magnesium turnings were mixed with no added solvent and refluxed on a steam bath for three hours; the hydrogen chloride by-product was run into a water trap. The excess acid chloride was removed by vacuum distillation, using a water pump, and the liquid residue was poured off the magnesium turnings, which in turn were rinsed with 75 cc. of ether. The ether washings were added to the reaction product and the mixture was chilled. Large granular crystals of the product formed in a solid mass. The weight of crude product was 135 Gm. (82% of theoretical). Recrystallization gave the pure compound in the same manner as previously stated, although for the syntheses described in this paper the crude product was suitable.

Anal.—Calcd. for $C_9H_8O_4NCl$: C, 47.1; H, 3.5. Found: C, 47.8; H, 4.0.

***p*-Nitrophenyl-β-dialkylaminopropionates.**—One-tenth mole of the β-chloroester was dissolved in 200 cc. of absolute ether to which was added 0.2 mole of the dialkylamine. The mixture was refluxed for fifteen minutes and chilled for a day or two in the refrigerator. In virtually all cases the by-product dialkylamine hydrochloride was filtered off in quantities indicating a yield of greater than 90% of theoretical. The ethereal filtrate was then dried over anhydrous sodium sulfate. To the dried solution was added an ethereal solution of hydrogen chloride. In general, the hydrochloride of the *p*-nitrophenyl-β-dialkylaminopropionate separated as a heavy oil that solidified into a crystalline mass after a few

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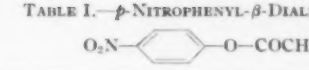
‡ Associate Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

TABLE I.—*p*-NITROPHENYL-β-DIALKYLAMINOPROPIONATES



R	Yields, %	M. P., ° C.	Formula	Chloride Analysis, % Calcd.	Found
C_2H_5[a]	66	136–137	$C_{12}H_{15}O_4N_2Cl$	11.71	11.54
C_3H_7[b]	73	116–118	$C_{14}H_{23}O_4N_2Cl$	10.71	10.91
n-C_4H_9[b]	89.5	96– 98	$C_{17}H_{27}O_4N_2Cl$	9.88	10.02
n-C_5H_{11}[a]	47	87– 89	$C_{19}H_{31}O_4N_2Cl$	9.16	8.72
iso-C_5H_{11}[a]	60	81– 82	$C_{19}H_{31}O_4N_2Cl$	9.16	8.95

[a] Acetone-ether used as recrystallizing solvent.
[b] Ethyl acetate used as recrystallizing solvent.

days at refrigerator temperatures. The products were recrystallized from acetone-ether mixtures or in some cases from ethyl acetate. The esters prepared are listed in Table I.

***p*-Aminophenyl-β-dialkylaminopropionates.**—The *p*-nitrophenyl-β-dialkylpropionates were reduced to the *p*-amino form in the following general manner: 2 Gm. of a 5% Pd-C catalyst, 50 cc. of absolute ethanol, and a sufficient amount of alcoholic hydrogen chloride to react with the newly created amino group were added to each 10 Gm. of the hydrochloride of the *p*-nitro compound. The mixture was reduced with hydrogen under an initial pressure of 40 lb. The reductions were rapid but were allowed to proceed for two hours. The filtrate from the reduced mixture was run directly into 350 cc. of anhydrous ethyl acetate, upon which a crystalline precipitate formed in most cases. If an oil formed it was allowed to stand in the refrigerator for a few days until the oil crystallized. The crystalline precipitates were recrystallized from alcohol or from mixtures of alcohol and ethyl acetate. The reduced compounds are described in Table II.

Phenyl-β-chloropropionate.—One mole of β-chloropropionyl chloride and one mole of phenol were placed in a reflux apparatus. The mixture was warmed on a steam bath for one hour; the fumes of the evolved hydrogen chloride were run into a water trap. The flask was then arranged for distillation and the contents were distilled, b.p. 148–149° at 24 mm., to give an almost quantitative yield of product. The literature reports boiling points of 132–135° at 12 mm. (3) and 141–143° at 18 mm. (4) for this compound. The product was used without further purification.

Phenyl-β-dialkylaminopropionates.—The general procedure for the preparation of the phenyl-β-dialkylaminopropionates was essentially the same as that described for the preparation of the *p*-nitrophenyl-β-dialkylaminopropionates. It consisted of treating 0.1 mole of phenyl-β-chloropropionate with 0.2 mole of the desired amine in ethereal solution. The by-product amine hydrochloride was removed by filtration, and the ethereal filtrate was treated with an excess of ethereal hydrogen chloride upon which the products crystallized readily. They were recrystallized from alcohol or from ethyl acetate. The esters prepared are listed in Table III.

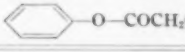
***p*-Acetylaminophenyl-β-chloropropionates.**—Ten grams of *p*-nitrophenyl-β-chloropropionate was dissolved in 120 cc. of absolute ethanol and placed in a conventional Parr low-pressure catalytic hydrogenator together with 4 Gm. of 5% palladium-on-carbon catalyst. The mixture was shaken under

TABLE II.—*p*-AMINOPHENYL-β-DIALKYLAMINOPROPIONATES

R	Yields, %	M. P., ° C.	Formula	Chloride Analysis, % Calcd.	Found
C_2H_5[a]	65	186–188	$C_{12}H_{22}O_2N_2Cl_2$	22.93	22.85
n-C_3H_7	54	199–200	$C_{13}H_{24}O_2N_2Cl_2$	21.03	20.87
n-C_4H_9	80	192–194	$C_{17}H_{36}O_2N_2Cl_2$	19.41	19.49
n-C_5H_{11}	80	196–198	$C_{15}H_{24}O_2N_2Cl_2$	18.03	18.02
iso-C_5H_{11}	78	198–200	$C_{15}H_{24}O_2N_2Cl_2$	18.03	18.04

[a] Compound recrystallized from alcohol. All others recrystallized from alcohol-ethyl acetate mixtures.

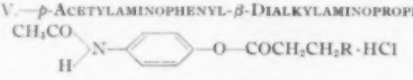
TABLE III.—PHENYL-β-DIALKYLAMINOPROPIONATES



R	Yields, %	M. P., ° C.	Formula	Chloride Analysis, % Calcd.	Found
—$N(C_2H_5)_2$[a, b]	65	86– 88	$C_{13}H_{20}O_2NCl$	13.76	13.90
—$N(n\text{-}C_3H_7)_2$[b]	66.5	140–143	$C_{15}H_{24}O_2NCl$	12.41	12.41
—$N(n\text{-}C_4H_9)_2$[b]	57	93– 95	$C_{17}H_{28}O_2NCl$	11.30	11.24
—$N(C_5H_{10})$ = (piperidino)[c]	74	204–206	$C_{15}H_{22}O_2NCl$	13.14	13.17

[a] Hygroscopic.
[b] Recrystallized from ethyl acetate.
[c] Recrystallized from ethyl alcohol.

TABLE IV.—*p*-ACETYLAMINOPHENYL-β-DIALKYLAMINOPROPIONATES



R	Yield, %	M. P., ° C.	Formula	Chloride Calcd.	Analysis, % Found
—N(C_2H_5)$_2$	63	180–182	$C_{15}H_{23}O_3N_2Cl$	11.27	11.29
—N(*n*-C_3H_7)$_2$	64	177–179	$C_{17}H_{27}O_3N_2Cl$	10.34	10.39
—N(*n*-C_4H_9)$_2$	89	181–182	$C_{19}H_{31}O_3N_2Cl$	9.56	9.55
—N(*n*-C_5H_{11})$_2$	55	155–156	$C_{21}H_{35}O_3N_2Cl$	8.88	9.09
—N(iso-C_5H_{11})$_2$	55	168–170	$C_{21}H_{35}O_3N_2Cl$	8.88	8.85
—N(C_5H_{10}) = (piperidino)	61	232–233	$C_{16}H_{23}O_3N_2Cl$	10.85	10.85

an initial pressure of 40 lb. of hydrogen for forty-five minutes, although the reduction appeared to be complete in about fifteen minutes. The catalyst was removed by filtration, and the filtrate was run directly into 750 cc. of ice-water mixture. The product separated as a white crystalline precipitate which was removed by filtration, washed with water, and dried. The weight was 6.7 Gm., corresponding to a yield of 77% of theoretical. It was difficult to purify the material by recrystallization because it decomposed rapidly in solution. The best product obtained had a melting point of 64–69°. In the dry state, the material can be stored in the refrigerator for a prolonged period of time.

Twelve grams of *p*-aminophenyl-β-chloropropionate was dissolved in 60 cc. of acetic anhydride in an ordinary Erlenmeyer flask and warmed on a steam bath for fifteen minutes. The hot mixture was poured with stirring into 500 cc. of ice-cold water to yield a crystalline mass of the acetylamino compound. This was allowed to stand at room temperature for several hours and then was filtered to obtain the crystalline product, which was dissolved in boiling water and allowed to crystallize several times. The weight of the final product was 8.7 Gm. of white crystals, m.p. 123–125°.

Anal.—Calcd. for $C_{11}H_{12}O_2NCl$: C, 54.6; H, 4.6. Found: C, 54.0; H, 4.9.

***p*-Acetylaminophenyl-β-dialkylaminopropionates.**—These compounds were prepared in the same manner as the *p*-nitrophenyl and phenyl compounds. One-tenth mole of *p*-acetylaminophenyl-β-chloropropionate was treated with 0.2 mole of the desired amine in ether solution. The only modification in the procedure was that a Soxhlet cup was used to hold the beginning material because of its limited solubility in ether, and the diamine and ether were placed in the lower flask. After several hours the *p*-acetylamino compound was completely extracted from the cup and the by-product amine hydrochloride formed in the lower flask. This was removed by filtration, and the filtrate was dried and treated with ethereal hydrogen chloride in the usual manner. The products were recrystallized from alcohol-ethyl acetate mixtures. The esters prepared are listed in Table IV.

SUMMARY

Several new phenyl esters of β-dialkylaminopropionic acids have been prepared. These, for the most part, include the phenyl, *p*-nitrophenyl, *p*-aminophenyl and *p*-acetylaminophenyl esters of diethyl, di-*n*-propyl, di-*n*-butyl, di-isoamyl, di-*n*-amyl, and piperidino propionic acids.

Of the above esters, the *p*-aminophenyl-β-dialkylaminopropionates have had a preliminary pharmacological testing and have exhibited a moderate antispasmodic activity. The other esters have not been tested but will be reported on by others upon completion of the testing.

REFERENCES

(1) Whitacre, F. M., *Anesthesia & Analgesia*, **18,** 112 (1939).
(2) Soine, T. O., and Wilson, C. O., unpublished report.
(3) Mayer, F., and Van Zutphen, L., *Ber.*, **57B,** 200(1924).
(4) Skraup, S., and Nieten, F., *ibid.*, **57B,** 1303(1924).

Further Studies on the Bioassay of *Veratrum viride**,†

By HOWARD J. JENKINS‡ and B. V. CHRISTENSEN§

The results of an attempt to employ the emetic response of the common pigeon to establish a biological unit of *Veratrum viride*, based on its therapeutic activity, are described. Interrelationships between the amounts of tinctures required to produce a 50 per cent response in pigeons and the amounts of these tinctures required to produce equivalent percentage maximum blood pressure reductions in both the dog and the human are found to obtain.

VERATRUM VIRIDE contains a number of alkaloids, some of which have now been identified and assayed for their hypotensive potencies (1–3) even though isolation of active constituents is rendered difficult because of the ease with which the principal alkaloids are hydrolyzed.

It is quite likely that these alkaloids, in the main, produce similar pharmacologic effects in animals and men. These effects are, however, not identical, as is shown by the actions elicited by the administration of the individual substances (1).

The need for a biological method of assay is clearly indicated if any given preparation of the drug is to be of reliable benefit. Even in the absence of a generally acceptable assay method, the stature of *Veratrum viride* as a hypotensive agent has been increasing recently.

Christensen and McLean employed a modification of the pigeon emesis method of Hanzlik (4) as a starting point in the development of an assay for *Veratrum viride*. They showed that this revised method was sufficiently accurate and produced sufficiently consistent results to be used as the basis for a procedure designed to evaluate the pharmacotherapeutic activity of this drug (5). In a later work some study of the correlation between the Minimum Emetic Dose (M. Em. D.) determined by the modified pigeon emesis method and per cent blood pressure fall in dogs and cats occasioned by the injection of Veratrum was made by Christensen and McLean.

Fries, Stanton, and Moister (1) report a systematic investigation of the activities of various chemical fractions of *Veratrum viride* in hypertensive humans.

A combination of the methods presented above suggests itself. To devise such a combination assay, some vital relationships must be worked out, these being the quantitative relationships applying to the effects of the preparation on various species of test animals, finally including man. In view of the potentialities and also the limitations of the method as proposed by Christensen and McLean, it seemed advisable to reinvestigate the possibility of the use of this assay, in modified form, to establish a biological unit of *Veratrum viride*.

The concept of the pigeon minimum emetic dose (M. Em. D.) of a preparation of *Veratrum viride* was changed to that of the pigeon median emetic dose of M. Em. D_{50}, which is that amount of the drug, expressed in cc. of tincture per Kg. body weight, which will produce emesis in 50 per cent of a group of pigeons within fifteen minutes after the intravenous injection of the diluted tincture. This M. Em. D_{50} was derived both graphically by the Litchfield-Wilcoxon method (6) and arithmetically by Behren's method of integration from the data obtained in the work.

EXPERIMENTAL

If the pigeon emetic doses of tinctures made from different sources of *Veratrum viride* produce consistently significant comparable blood pressure reductions in humans, a biological unit of potency of *Veratrum viride* based on the pigeon M. Em. D_{50} may be evolved. This unit could be used as the unit of potency in the assay of *Veratrum viride* and also as a measure of therapeutic dosage of tinctures of *Veratrum viride* based on the needs of the patient. The following experiments were designed to determine whether or not such comparable relationships are indicated.

A mixture of the more commonly occurring varieties of pigeons of both sexes were used in the assay. Adult pigeons weighing approximately 300–600 Gm. were selected. All injections were made into the brachial vein. The vomiting of the pigeons is an unmistakable physiological phenomenon. Depending upon whether or not the bird has recently ingested food and water, the retching act may or may not be productive. For the purposes of the assay it makes no difference either way. The bird need only "go through the motions" to have the result labeled positive.

All lots of powdered *Veratrum viride* used were confirmed according to N. F. VIII and all tinctures were prepared according to N. F. VIII specifications. The following results show the comparable relationships between two tinctures prepared from different lots of *Veratrum viride* obtained from dif-

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† Abstract of dissertation presented to the Graduate School, Ohio State University, by Howard J. Jenkins in partial fulfillment of the requirements for the degree of Doctor of Philosophy, December, 1950.

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§ Dean, College of Pharmacy, Ohio State University.

ferent sources. They are labeled tincture H and tincture C.

Data (together with their integration by Behren's method) showing the effect of tincture H in varying doses (dilution 1:10) on 48 pigeons weighed to the nearest 5 Gm. are shown in Table I.

The M. Em. D_{50} of tincture H in doses at equal logarithmic intervals and computed from integrated data is 0.222 cc. dil. tincture/Kg. (see Fig. 1).

TABLE I.—EMETIC RESPONSE OF ARITHMETICALLY SPACED DOSES OF TINCTURE H

Dose in Cc. Dil. Tr./ Kg.	Emesis	No Emesis	Integrated Data[a] Emesis	Integrated Data[a] No Emesis	Integrated Data[a] Frequency
0.310	7	1	24	1	24/25
0.280	4	4	17	5	17/22
0.250	5	3	13	8	13/21
0.230	4	4	8	12	8/20
0.210	3	5	4	17	4/21
0.180	1	7	1	24	1/25
			67	67	

[a] The M. Em. D_{50} of tincture H as obtained from this integrated data is 0.239 cc. dil. tincture/Kg.

The M. Em. D_{50} of tincture C administered by equal logarithmic intervals and computed from integrated data is 0.098 cc. dil. tincture/Kg. (see Fig. 2 and Table II).

In the experimental work involving the effect of the tincture of *Veratrum viride* on the circulation of the dog, the systolic and diastolic pressures were measured by the use of the combination of a Statham transducer, a control box, and an electrocardiograph.

Data showing the maximum effects of tincture H (dilution 1:10) in various doses on the blood pressure of the same dog (anesthetized with veterinary Nembutal sodium) are shown in Table III and Fig. 3.

Data showing the maximum effects of tincture C (dilution 1:10) in various doses on the blood pressure of the same dog (anesthetized with veterinary Nembutal sodium) are shown in Table IV and Fig. 4.

Data showing the maximum effects of the median emetic dose of tincture H on the blood pressure of the human (female: age 41 years) with uncomplicated benign essential hypertension are shown in Table V.

Data showing the maximum effects of the median emetic dose of tincture H on the blood pressure of

TABLE II.—EMETIC RESPONSE OF ARTHMETICALLY SPACED DOSES OF TINCTURE C

Dose in Cc. Dil. Tr./ Kg.	Emesis	No Emesis	Integrated Data[a] Emesis	Integrated Data[a] No Emesis	Integrated Data[a] Frequency
0.180	7	1	24	1	24/25
0.130	6	2	17	3	17/20
0.100	5	3	11	6	11/17
0.080	2	6	6	12	6/18
0.060	4	4	4	16	4/20
0.040	0	8	0	24	0/24
			62	62	

[a] The M. Em. D_{50} of tincture C as obtained from this integrated data is 0.090 cc. dil. tincture/Kg.

TABLE III.—MAXIMUM CIRCULATORY EFFECTS OF VARIOUS DOSES OF TINCTURE H (INCLUDING PIGEON MEDIAN EMETIC DOSE)

Dose in Cc. Dil. Tr./Kg.	Maximum Reduction, % Systolic	Diastolic	Heart Rate
0.169	21.70	41.11	28.00
0.239[a]	35.41	51.72	41.66
0.338	43.75	56.66	27.27
0.478	58.82	76.19	50.00

[a] Pigeon median emetic dose of tincture H.

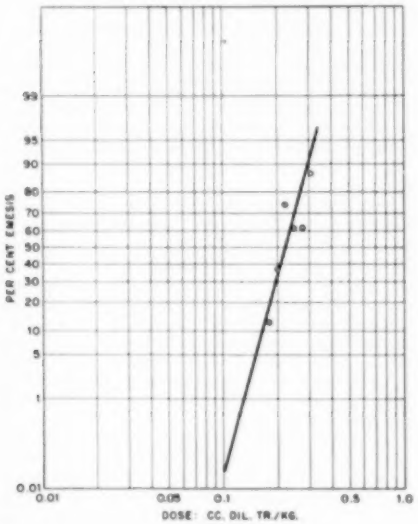


Figure 1

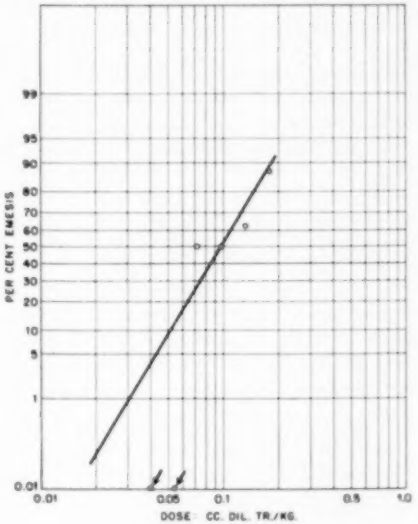


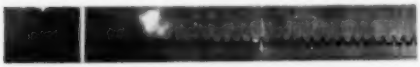
Figure 2

the human (female: age 43 years) with uncomplicated essential hypertension are shown in Table VI.

TABLE IV.—MAXIMUM CIRCULATORY EFFECTS OF VARIOUS DOSES OF TINCTURE C IN THE DOG (INCLUDING PIGEON MEDIAN EMETIC DOSE)

Dose in Cc. Dil. Tr./Kg.	Maximum Reduction, %		
	Systolic	Diastolic	Heart Rate
0.063	22.34	37.50	31.03
0.090[a]	34.40	51.85	29.62
0.126	45.87	58.85	39.78
0.180	56.17	70.84	33.33

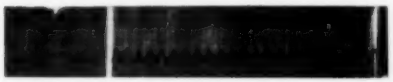
[a] Pigeon median emetic dose of tincture C.



Normal

Maximum Reduction

Fig. 3.—Maximum effects of tincture H in various doses on the blood pressure of the same dog.



Normal

Maximum Reduction (Systolic)

Maximum Reduction (Diastolic)

Fig. 4.—Maximum effects of tincture C in various doses on the blood pressure of the same dog.

TABLE V.—MAXIMUM CIRCULATORY EFFECTS OF THE PIGEON MEDIAN EMETIC DOSE OF TINCTURE H IN THE HUMAN

Dose in Cc. Tr./Kg. Orally	Maximum Reduction, %	
	Systolic	Diastolic
0.0239	8.55	4.44

TABLE VI.—MAXIMUM CIRCULATORY EFFECTS OF THE PIGEON MEDIAN EMETIC DOSE OF TINCTURE H IN THE HUMAN

Dose in Cc. Tr./Kg. Orally	Maximum Reduction, %	
	Systolic	Diastolic
0.0239	9.33	6.25
0.0239	8.57	7.14

CONCLUSIONS

1. Pigeon emesis represents a sharp end point response; hence, effects in terms of pigeon emesis from equal doses are quantitatively reproducible.

2. There is a quantitative relationship between the pigeon emetic dose and blood pressure response in dogs, e. g., increase in dose produces a corresponding increase in blood pressure reduction.

3. The same relationship was demonstrated in blood pressure response in humans as in dogs.

4. There is, therefore, a quantitative relationship in the comparative effects of equal doses of tincture of *Veratrum viride* on the emetic center of the common pigeon and the circulatory systems of both the dog and the human.

5. Therefore the pigeon emetic response appears to be a measure of the therapeutically active principles of *Veratrum viride*.

REFERENCES

(1) Fries, E. D., Stanton, J. R., and Moister, F. C., *J. Pharmacol. Exptl. Therap.*, **98,** 166(1950).
(2) Krayer, O., and Acheson, G. H., *Physiol. Rev.*, **26,** 383(1946).
(3) Jacobs, W. A., and Sato, Y., *J. Biol. Chem.*, **181,** 55 (1949).
(4) Hanzlik, P. J., *J. Pharmacol. Exptl. Therap.*, **35,** 363(1929).
(5) Christensen, B. V., and McLean, A. P., THIS JOURNAL, **28,** 74(1939).
(6) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **95,** 99(1949).

Determination of Heavy Metals in Pharmaceutical Chemicals*

By FREDERICK N. STEWART and CLIFTON W. STRODE, Jr.

The official heavy metals tests have been criticized on a number of points, among which are failure to compensate for sample color, difficulty in the visual discrimination of extremely light colors, and nonuniformity of conditions. A scheme is suggested for standardizing conditions, with provision for sample color compensation through photoelectric measurements. The method essentially involves preparation of the sample solution by treatment according to functionality and solubility of the substance, adjustment of the *p*H to between 3 and 4 before addition of hydrogen sulfide, and photoelectric measurement with compensation for a sample blank. Accuracy of recoveries within 2 p.p.m. of added lead in final concentrations from 1 to 30 p.p.m. have been obtained with 37 typical pharmaceutical chemicals.

THE METHODS specified by the official compendia (1, 2) for the detection of heavy metal contamination in pharmaceutical chemicals have been criticized by several investigators (3–5). In the sulfide method, any color contributed by the substance being tested is counted as a heavy metal. Variations in the acidity of the final solutions result in different criteria of the heavy metal specification for different products. A large element of personal interpretation is involved in resolving the difficulties encountered in visual discrimination between extremely light colors, between different colors, and between color and turbidity. McArthur (3) has suggested an ingenious superimposition technique for the compensation of sample color, but it suffers from low sensitivity and retains the limitations of visual comparison. The dithizone test (4) employs a relatively unstable reagent, highly sensitive to trace contamination, in a comparatively involved procedure. It is useful, however, with products such as ferric and manganese glycerophosphates which are not conveniently adaptable to the sulfide method.

Bundy and Edman (5) demonstrated the difficulty in matching certain differently colored metal sulfides with lead sulfide and suggested that turbidity might prove a better criterion. The lead standard is justified, however, because other metals are either less common, less toxic, or, like arsenic, are provided for by specific tests where necessary.

In the method described herewith the substance to be tested is treated according to its position in an arbitrary classification based upon solubility and functionality, with provision for obtaining a solution at a *p*H of between 3 and 4 before the addition of hydrogen sulfide. Acetone is employed to increase the solubility of certain organic compounds and to decrease the solubility of the metal sulfides. Sample color is compensated for by measuring the absorbancy of a sample solution containing hydrogen sulfide against one without hydrogen sulfide. Adequate instrumental sensitivity to both color and turbidity is insured by the use of a blue filter and a relatively long light path.

* Received August 31, 1951, from the John F. Queeny Plant, Monsanto Chemical Company, St. Louis, Mo.
Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.

METHOD

Apparatus

Both model 402-E and model 450 Lumetron photoelectric colorimeters (6) have been used in this laboratory. While other colorimeters of comparable sensitivity might be employed, the model 450 Lumetron uses 100-ml. Nessler tubes etched for a light path of 150 mm. and is well suited for absorbancy measurements in light-colored solutions. Frequency variations in the power supply to this laboratory rendered regulation by the constant voltage transformer supplied with the instrument unsatisfactory in line operation, but good stability of readings was obtained with an 8-volt storage battery.

Reagents

Analytical reagent grade chemicals and U. S. P. test solutions are used where applicable. Some samples of reagent grade acetone have been found to produce a faint opalescence upon dilution and acidification. Simple distillation removed this anomalism. Hence, purchased acetone is redistilled to preclude spectrophotometric interference.

Preparation of Sample

Treat 4 Gm. of sample by the procedure under the following classification which best describes the substance, adjusting the final volume of solution in a 100-ml. graduated cylinder:

1. Weak Acids.—Dissolve in 50 ml. of acetone and dilute to 100 ml. with water.

2. Water-Soluble Neutral Compounds.—Dissolve in 40 ml. of water, add 8 ml. of 6% acetic acid, 50 ml. of acetone, and dilute to 100 ml. with water.

3. Salts of Weak Acids.—Dissolve or moisten with a few milliliters of water, add normal hydrochloric acid equivalent to the cation, followed by 50 ml. of acetone, and dilute to 100 ml. with water.

4. Compounds Requiring *p*H Adjustment (Strong Acids, Bases, or Buffers).—Dissolve or slurry in 40 ml. of water. Adjust to a *p*H of 3.5 by titrating with 10% hydrochloric acid or concentrated ammonium hydroxide to a spot indication on Hydrion paper,[1] or, preferably, with the aid of a glass electrode *p*H meter. Add 50 ml. of acetone and dilute to 100 ml. with water.

5. Acetone-Soluble Neutral Compounds.—Dissolve in 50 ml. of acetone, add 8 ml. of 6% acetic acid, and dilute to 100 ml. with water.

6. Difficultly Soluble Compounds.—If a powder, moisten with 20 ml. of 10% hydrochloric acid and heat over a steam bath for five minutes. Cool to 20°, allow to stand until the supernatant liquid is clear, filter by suction on a Hirsch funnel, and wash with a small amount of water. If an oil, extract with 20 ml. of 10% hydrochloric acid and filter the extract through a small circle of wet filter paper. Add two drops of 0.1% *p*-nitrophenol indicator solution to the filtrate or extract and titrate to a faint yellow end point with concentrated ammonium hydroxide. Add 8 ml. of 6% acetic acid, 50 ml. of acetone, and dilute to 100 ml. with water.

7. Deeply Colored Organic Compounds and Those Which React Chromatogenically with Hydrogen Sulfide.—Moisten, in a 600-ml. beaker, with 8 ml. of concentrated sulfuric acid. Grasp with beaker tongs and swirl until any initial reaction subsides. With continued swirling, heat to boiling over a moderate flame to carbonize. Remove flame and add 30% hydrogen peroxide solution drop by drop from a 15-ml. pipette, allowing the reaction to subside somewhat between drops. When there is a noticeable diminution in the vigor of the reaction on addition of hydrogen peroxide, heat cautiously to fumes, remove heat, and resume the addition of hydrogen peroxide until the solution is colorless. Boil to remove excess peroxide, cool the beaker in ice, add 10 ml. of water and two drops of 0.1% *p*-nitrophenol. Neutralize to a faint yellow end point with concentrated ammonium hydroxide, add 8 ml. of 6% acetic acid, and dilute to 100 ml. with water.

8. Volatile Liquids.—Evaporate on a steam bath. Dissolve the residue in 8 ml. of 6% acetic acid, add 50 ml. of acetone, and dilute to 100 ml. with water.

Determination of Heavy Metals

Pour 50 ml. of the prepared sample solution into a second 100-ml. graduated cylinder. To each portion add an additional 25 ml. of acetone.[2] Verify the *p*H with Hydrion paper or a *p*H meter, adjusting to 3.5 if necessary. To one portion add 20 ml. of saturated hydrogen sulfide water and dilute both solutions to 100 ml. with water.

After exactly ten minutes, measure the absorbancy of 150 mm. of the solution containing hydrogen sulfide against an equal depth of the blank solution, using a broad band filter with a peak transmission at 420 mμ.

Read the heavy metals content from a calibration curve plotted from absorbancy data on solutions prepared by adding aliquots of U. S. P. standard lead solution to mixtures of 50 ml. of acetone, 20 ml. of water, and 4 ml. of 6% acetic acid, adding 20 ml. of saturated hydrogen sulfide water, and diluting to 100 ml. with water.

Alternatively, measure each sample solution against the 15 p. p. m. (30 μg./2-Gm. sample) standard and refer to an absorbancy ratio calibration. The reading against the solution containing hydrogen sulfide will then give the heavy metals content in terms of current official specifications while the blank reading will illustrate the indicated correction.

[1] Micro Essential Laboratory, Brooklyn, N. Y.

[2] In isolated cases it may be necessary to modify the acetone concentration at this point in order to insure a clear solution.

EXPERIMENTAL

Absorbancies of two concentrations of lead sulfide solutions in 50% acetone, 150 mm. in depth, with the set of broad band filters supplied with the model 450 Lumetron colorimeter are shown in Table I.

TABLE I.—ABSORBANCIES OF LEAD SULFIDE SOLUTIONS

Filter	30 μg. Pb	60 μg. Pb
B-390	0.072	0.146
B-420	0.128	0.260
B-490	0.085	0.170
B-530	0.068	0.140
B-580	0.047	0.100
B-650	0.032	0.065

Typical calibration curves with the B-420 filter are illustrated in Fig. 1. The absorbancy ratio technique was suggested by the work of Hiskey, *et al.* (8).

The applicability of the method was verified by analyzing a number of representative materials. Known amounts of standard lead solution equivalent to 5 and 10 p. p. m., respectively, were added to additional portions of each material, and these portions were then analyzed. The results are listed in Table II. The column headed "Apparent" represents measurement against a lead standard with no correction for inherent sample color. The value is analogous to one which would be obtained through photoelectric interpretation of the official U. S. P. method. All other values are corrected.

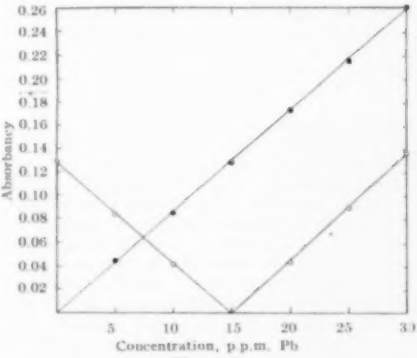


Figure 1.

●—Normal calibration curve for 2.0-Gm. sample.
○—Ratio calibration curve for 2.0-Gm. sample.

TABLE II.—RESULTS, AS Pb, OF THE DETERMINATION OF HEAVY METALS IN TYPICAL PHARMACEUTICAL CHEMICALS AND WITH KNOWN AMOUNTS OF LEAD ADDED

Sample	Method of Preparation	Apparent Pb	Corrected Pb		
		P. P. M.	P. P. M.	P. P. M. +5	P. P. M. +10
Acetanilid	5	4	4	8	13
Acetic anhydride	8	1	1	6	12
Acetophenetidin	5	2	2	7	13
Acetyl salicylic acid	1	2	1	5	10
p-Aminosalicylic acid	1	24	3	8	14
Ammonium salicylate	3	9	5	9	14
Benzoic acid	1	12	5	9	14
Caffeine	2	5	1	6	12
Caffeine citrate	4	25	16	21	27
Calcium chloride	2	15	11	15	19
Calcium glycerophosphate	4	24	16	21	25
Chloramphenicol	5	30	11	15	20
Glycerin	2	3	2	6	11
Glycerophosphoric acid	4	7	1	6	11
Magnesium glycerophosphate	4	33	25	29	34
Magnesium salicylate	3	9	7	13	19
Manganese chloride	2	7	3	8	12
Methyl salicylate	6	12	11	16	22
Phenolphthalein	6	12	9	14	19
Phenylacetamide	5	6	3	9	13
Phthalyl sulfathiazole	6	12	10	15	21
Potassium glycerophosphate	4	5	2	6	10
Saccharin	4	8	6	10	16
Saccharin sodium	3	4	2	8	13
Salicylic acid	1	10	7	11	15
Sodium acetate	3	7	3	9	13
Sodium benzoate	3	4	2	8	14
Sodium carbonate	4	5	2	7	12
Sodium gentisate	7	5	0	5	10
Sodium glycerophosphate	4	3	2	7	13
Sodium salicylate	3	4	2	7	13
Strontium salicylate	3	20	5	9	14
Succinyl sulfathiazole	6	12	11	15	20
Sulfanilamide	5	6	2	7	12
Sulfathiazole	5	6	0	6	10
Thenylpyramine HCl	4	8	3	7	12
Theobromine	6	4	1	5	9

These data represent analytical results obtained with 37 different chemicals by triplicate determinations, two of which incorporate known values from which deviations may be taken. The standard deviation, $s = \sqrt{\Sigma d^2/n}$, calculated from these 74 individual determinations is 0.75 p. p. m. Rounding off the $3s$ (99.73%) confidence limit to the nearest whole p. p. m. illustrates that recoveries of added lead up to 10 p. p. m. would be made with an accuracy of 2 p. p. m. in over 99% of the determinations.

DISCUSSION

In the official methods (1, 2) the sample preparation for each pharmaceutical is specified in the individual monograph. In some cases the metal sulfides are formed in mineral or acetic acid solution by the addition of hydrogen sulfide solution, while in other cases an alkaline solution and sodium sulfide reagent are specified. If the mineral acidity is too high, heavy metal sulfide formation is retarded or inhibited, and low results are obtained. In alkaline solution iron will react and be measured as a heavy metal, whereas in acid solution it will not. Since it is desirable to exclude iron from a toxic heavy metals test, the conditions of the proposed method were designed to have the *p*H of all sample solutions between 3 and 4.

For adjusting the *p*H of the solution, Evers (7) reported that bromphenol blue had a colorless plateau in the desired range. We could not duplicate his results. *p*-Nitrophenol is colorless at a *p*H of about 5, and the addition of acetic acid at this point will bring the solution within the desired range, providing no buffers are present. In the latter case the addition of mineral acid and an external indicator, preferably a *p*H meter, are required.

The only substances encountered which did not fit readily into the proposed scheme of sample preparation were ferric glycerophosphate and manganese glycerophosphate. Various reducing and complexing agents tried were of little avail toward enabling the measurement of heavy metal sulfides in the presence of these chemicals. A measure of success was achieved by resorting to an isolation technique which was also designed as an alternate to the wet ashing procedure. This technique involved extraction of the heavy metals with dithizone, followed by destruction of the dithizone with perchloric acid and development of the sulfides in a solution of the residue. The advantages over the standard lead limit test (4) were, however, deemed insufficient to warrant inclusion in the proposed method. Experience with materials such as sodium gentisate (Table II) demonstrated that the wet ashing technique affords excellent recoveries when properly carried out.

SUMMARY

1. The degree to which sample color affects the interpretation of heavy metal determinations by the current official sulfide method has been demonstrated.

2. A scheme is proposed for unification of the conditions of the test and its adaptation to photoelectric colorimetry, providing for blank compensation, simplicity, and adequate sensitivity for the purpose intended.

REFERENCES

(1) "United States Pharmacopeia," Fourteenth Revision Mack Publishing Co., Easton, Pa., 1950.
(2) "The National Formulary," 9th ed., Mack Publishing Co., Easton, Pa., 1950.
(3) McArthur, E. A., THIS JOURNAL, **38**, 321(1949).
(4) Bambach, K., *Ind. Eng. Chem., Anal. Ed.*, **12**, 63 (1940).
(5) Bundy, C. H., and Edman, W. W., THIS JOURNAL, **35**, 115(1946).
(6) Photovolt Corp., 95 Madison Ave., New York 16, N. Y., technical bulletins.
(7) Evers, N., *Chemist and Druggist*, **93**, 1034(1920); *Pharm. J.*, **105**, 85(1920).
(8) Hiskey, C. F., *Anal. Chem.*, **21**, 1440(1949); Hiskey, C. F., Rabinowitz, J., and Young, I. G., *ibid.*, **22**, 1464 (1950).

The Manufacture of Compressed Soluble Tablets*

By G. J. SPERANDIO and H. G. DeKAY*

Using lactose as a base for soluble tablets, experiments with different granulating agents and lubricants were made in an effort to obtain a practical working formula. Additional reports include findings on the relationship of mesh-size of granulation to ease of compression and amount of lubricant required for compression. A formula and procedure for soluble compressed tablets is given and two soluble lubricants are suggested.

EVER since the introduction of tablet triturates to the profession (1), pharmacy has been interested in soluble tablets and their manufacture by tablet machines. The original soluble tablets were made by molding, and consisted simply of a base such as lactose containing soluble medication. Tablets made by this method were ideal for use as hypodermic tablets and today most hypodermic tablets are still made by a modified molding procedure.

With the advent of the antibiotics, soluble tablets similar to those used for hypodermic medication have become a popular form of dosage. Very small, rapidly soluble, and easily disintegrated, these tablets are recommended for administration in infant formulas, milk, or similar liquids, and in some instances are placed directly under the tongue to be dissolved. Most of these tablets have been made by molding rather than by compression on a tablet machine. This method of manufacture is used because lactose, the most common base, cannot normally be compressed in a tablet machine without adding to it a lubricant to facilitate compression; and since most of the good lubricants are insoluble, a completely soluble tablet would not be obtained.

Soluble lubricants have been reported from time to time. Boric acid (2, 5), sodium chloride (3), and mixtures of soluble powders (4) have been used for specific tablets but have not proved satisfactory in all cases. One disadvantage of most soluble lubricants, boric acid in particular, is that many times a high percentage of the lubricant is necessary for compression.

The purpose of this work was to investigate the possibilities of making compressed soluble tablets from lactose granulations and to find a soluble lubricant which would yield a satisfactory tablet both from a physical and a therapeutic standpoint. Experiments were limited to one base—lactose—because of its ideal solubility, therapeutic inertness, and low cost.

EXPERIMENTAL

Two important factors contributing to the compressibility of a granulation are the hardness of the granules and the percentage of fine powders present. Since both of these properties might be imparted to a granulation by the relative amount of adhesive agent in it, granulations were made using varying percentages and varying strength solutions of granulating agents, so that the best adhesive and the optimum percentage of adhesive for lactose granulations might be found. The following granulations were made:

(A) Thirty-nine different formulations with varying strength solutions of gums and mucilaginous substances:

* Received May 5, 1950, from the Research Department, Purdue University, School of Pharmacy, Lafayette, Ind.
Presented to the Practical Pharmacy Section, A. PH. A., Atlantic City meeting, May, 1950.

Acacia mucilage, 4–35% strength
Tragacanth mucilage, 1–5% strength
Pectin gel, 1–5% strength
Chondrus mucilage, 1–3% strength
Karaya mucilage, 1–4% strength
Quince seed mucilage, 1–4% strength
Gelatin solution, 1–5% strength
Agar suspension, 0.25–1% strength

(B) Thirty different formulations with varying strength solutions of sugars:

Sucrose solution, 2–50% strength
Maltose solution, 2–30% strength
Fructose solution, 2–25% strength
Liquid glusose solution, 2–25% strength
Sorbose solution, 2–25% strength

(C) Thirteen different formulations with varying strength solutions of cellulose compounds and sodium alginate:

Methylcellulose, 15 c. p. s. solution
Methylcellulose, 100 c. p. s. solution, 1% strength
Methylcellulose, 400 c. p.s. solution
Methylcellulose, 1,500 c. p. s. solution
Hydroxyethyl cellulose, 1 and 2% solutions
Carboxymethyl cellulose, 1 and 2% solutions
Sodium alginate solution, 0.25–2%

Each of these granulations was made in the same manner: powdered lactose was mixed by hand with a solution of the granulating agent, then pushed through a 20-mesh screen and dried overnight in air. The dried granulation was again pushed through a 20-mesh screen and compression was attempted on a Stokes single-punch tablet machine, using a $^{3}/_{16}$-in. die and running at the rate of 90 tablets a minute. After each attempt at compression, the tablet machine was cleaned before a new granulation was run. It should be emphasized that the fundamental idea was not to find a granulation which would run freely by itself, but rather to make comparative tests on the relative ease of compression of the various granulations and determine which one would compress with a minimum of difficulty.

It was found that, of all the gums and mucilaginous substances tried, acacia and gelatin seemed to be the only substances showing promise of making a good lactose granulation, but neither of these was entirely satisfactory because of capping and binding in the die. Of all the sugars tried, it was found that a 2% solution of liquid glucose offered definite advantages over all other granulating agents in preparing a compressible granulation. This confirms previous work done along the same line (5). Most of the granulations made with sugars as the adhesive agents caused trouble by sticking to the surfaces of the punches. The granulations made with cellulose compounds as the adhesive agents varied in compressibility according to the type of cellulose compound used. Granulations made with 1% solutions of methylcellulose 15, 100, and 400 c. p. s. all evidenced slight capping and binding but were compressible. Granulations made with a 1% solution of methylcellulose 1,500 c. p. s. compressed very well with only slight sticking to the punches but tablets were very slow to disintegrate.

Additional granulations of lactose were made using solutions of liquid glucose in strengths varying from 0.5 to 4% and each granulation was compressed as before. It was found that a 2% solution of liquid glucose was the granulating agent of choice for lactose, and that the best granulation was obtained by adding this solution to lactose in the ratio of 20 cc. for every 100 Gm. of lactose.

Since soluble tablets are usually very small and seldom weigh more than 1 grain, a smaller size granulation must be used than is normally needed for regular compressed tablets. Experiments were conducted to determine the optimum size granule for small tablets and to ascertain what effect the granule size has on the ease of compression of a granulation.

Granulations of lactose were made with the following granulating agents:

Solutions of liquid glucose 1, 2, 5, 10, and 20%
Mucilages of acacia 4, 10, and 20%
2% solution of gelatin
1% solutions of methylcellulose, 15 and 1,500 c. p. s.

Each of these granulations were divided into portions and prepared in mesh sizes ranging from 16-mesh to 80-mesh. Compression was then attempted on the Stokes single-punch tablet machine as in the previous experiments. Results of these compressions indicated that a definite relationship exists between the compressibility of a granulation and the granule size. In every instance tried, as the size of the granules was decreased, the granulation became more difficult to compress without a lubricant. All granulations, even those which compressed poorly, evidenced more compressibility in a 20-mesh size than in any smaller size.

To confirm this theory, additional portions of the standard lactose granulation were compressed with substances known to be good tablet lubricants. Samples of the lactose granulation were prepared ranging in granule size from 16-mesh to 100-mesh. Each granulation was then mixed with varying percentages of talc and of magnesium stearate and tumbled in a closed container until uniformly mixed. Increasing amounts of the lubricant were added to each granulation until satisfactory compression was achieved, thus determining the minimum amount of lubricant needed for satisfactory compression of each mesh-size granulation. The following results were obtained:

(*a*) 16-mesh granulation required 3% talc for satisfactory lubrication.

(*b*) 20-mesh granulation required 3% talc for satisfactory lubrication.

(*c*) 30-mesh granulation required 5% talc for satisfactory lubrication.

(*d*) 40-, 60-, 80-, and 100-mesh granulations were not satisfactorily lubricated with 5% talc.

(*e*) 16- and 20-mesh granulations required 2% magnesium stearate for satisfactory lubrication.

(*f*) 30-mesh granulation required 5% magnesium stearate for satisfactory lubrication.

(*g*) 40-, 60-, 80-, and 100-mesh granulations were not lubricated satisfactorily by 5% magnesium stearate.

In the above cases no more than 5% lubricant was added to any granulation, this being considered the maximum amount of lubricant allowable for a good granulation. With both lubricants, granula-

tions smaller than 30-mesh would not compress satisfactorily with 5% lubricant, while the 16-mesh and 20-mesh granules compressed quite well with 3% talc and with 2% magnesium stearate. 20-Mesh granulation is to be preferred over the 16-mesh for small tablets because it fills the dies more evenly and makes possible the compression of tablets more uniform in weight.

The following substances were tested for lubricating action on the lactose granulation:

Aminoacetic acid	Potassium phosphate
Ammonium chloride	Sodium benzoate
Ammonium citrate	Sodium bicarbonate
Ammonium phosphate	Sodium chloride
Citric acid	Sodium citrate
Magnesium chloride	Sodium lactate
Magnesium sulfate	Sodium lauryl sulfate
Potassium benzoate	Sodium oleate
Potassium chloride	Sodium stearate
Potassium citrate	Sodium sulfate

Each compound was mixed with the granulation in amounts ranging from 1 to 5% by weight, and compression was attempted on the Stokes single-punch tablet machine. Four substances offered evidence of lubrication to the granulation: sodium benzoate, sodium oleate, sodium stearate, and sodium lauryl sulfate. The best lubrication was obtained when these substances were present in the granulation to the extent of 2%, except for sodium stearate which gave good lubrication in a 1% proportion. However, all of the tablets made with these lubricants had a slow disintegration time (three minutes) and did not dissolve to give a completely clear solution. Of all the tablets tested, those made with sodium benzoate as a lubricant were the best, dissolving in two minutes to give a relatively clear solution.

In literature of the Carbon and Carbide Chemical Corporation, the "Carbowax" compounds are advocated for use as water-soluble waxes. Examination of samples showed them to be of smooth nature, soluble in water, and their suitability as tablet lubricants was investigated. Lactose granulations were made and samples of "Carbowax 1500" and "Carbowax 4000" were added both in varying strength solutions and in powdered form. It was found that "Carbowax 4000" powdered to a 30-mesh size gave excellent lubrication to the granulation when added in the amount of 4%. Tablets thus made were found to be of excellent appearance with a solubility time of approximately two minutes, forming a clear solution.

Theorizing that the degree of fineness of the lubricant might affect the speed of disintegration of the tablets, attempts were made to determine the optimum mesh-size lubricant for the lactose granulation. Accordingly, samples of the "Carbowax 4000" were made in varying degrees of fineness by pushing them through screens ranging in size from 30-mesh to 80-mesh. Samples of the granulation were then mixed with portions of the powdered lubricant in increasingly large amounts until satisfactory compression was attained. The minimum amount of each mesh-size lubricant needed for satisfactory compression was determined and the resulting tablets were tested for speed of disintegration and solubility. The results are shown in Table I.

From these results it would appear that a smaller amount of a powdered lubricant is needed when it is in a finer state of subdivision, and that the disintegration time of the tablet is decreased as the particle size of the lubricant is decreased. Also, in the case of "Carbowax 4000," a 60-mesh powder offers more rapid disintegration to the tablet than other mesh sizes of the same compound. Possibly this compound may be used as a soluble lubricant for compressed hypodermic tablets since it has been reported to have a relatively low toxicity (6, 7).

TABLE I.—DISINTEGRATION AND SOLUBILITY OF TABLETS COMPRESSED WITH DIFFERENT MESH-SIZE LUBRICANTS

Mesh Size of Lubricant	Minimum Needed for Lubrication, %	Disintegration Time of Tablets	Solubility Time of Tablets
30	4	3 min.	4 min.
40	3	23 sec.	1 min.
60	2	17 sec.	45 sec.
80	2	40 sec.	70 sec.

Compression of the lactose granulation was next attempted on the rotary tablet machine, using powdered "Carbowax 4000" as a lubricant. A trial lot of 3,000 tablets was made using $^3/_{16}$ in. punches, the weight of each tablet being 1 grain. Compression was satisfactory and the machine ran without difficulty, but a slight sticking to the surfaces of the upper punches was noticed. After eliminating all other possible causes of sticking, it was found that the degree of pressure used during compression materially affects both the degree of sticking and the rate of disintegration of the finished tablet. By carefully adjusting the pressure on the tablet machine to yield a firm tablet with a hardness of 2 Kg. as measured by the Monsanto hardness tester, a tablet was obtained which did not stick and which disintegrated in thirty seconds. Small particles of the Carbowax remained in suspension, requiring an additional fifteen seconds to dissolve.

Since previous experiments had indicated that sodium benzoate might also be a satisfactory lubricant for the lactose granulation, additional tests using it as a lubricant were made. According to Scoville (8), sodium acetate increases the solubility of sodium benzoate. Different portions of the lactose granulation were lubricated with varying mixtures of these two powdered substances and compressed. It was found that the best lubrication was offered by a mixture of 4% sodium benzoate and 1% sodium acetate.

The formulas and procedures for compressed soluble tablets may be written as follows:

Ingredients	Tablets Containing Less Than $^1/_4$ Grain Medication	Tablets Containing More Than $^1/_4$ Grain Medication
Medication	Required amount	Required amount
Lactose	*q.s.* to 0.5 gr. per tablet	*q.s.* to 0.9 gr. per tablet
2% Solution of liquid glucose	20 cc./100 Gm.	20 cc./100 Gm.

Calculate the number of tablets desired and multiply the amounts of ingredients by that number. Mix the medication with the lactose thoroughly and granulate by hand with the glucose solution. Push through a 20-mesh screen and dry in air. Rescreen through a 20-mesh sieve and weigh. Add either of

the following lubricants: 4% sodium benzoate 60-mesh and 1% sodium acetate 60-mesh, or 2% powdered "Carbowax 4000" (60-mesh). Mix well by tumbling and compress.

SUMMARY AND CONCLUSIONS

1. The most suitable granulation for compressed soluble tablets using lactose as a base is made by granulating lactose with a 2 per cent solution of liquid glucose.

2. Lactose granulations made with sugars as the adhesives compress more readily and disintegrate more quickly than those made with gums.

3. The amount of lubricant required for a lactose granulation increases as the granule size decreases.

4. A 20-mesh granule is the optimum size for making compressed tablets weighing one grain or less.

5. Smaller amounts of a lubricant are needed when the lubricant is in a very fine powder, and the disintegration time of the tablet is decreased as the particle size of the lubricant is decreased.

6. A mixture of 4 per cent sodium benzoate and 1 per cent sodium acetate as a 60-mesh powder is a satisfactory soluble lubricant.

7. Powdered "Carbowax 4000" in a 60-mesh size is a satisfactory soluble lubricant for tablet granulations.

REFERENCES

(1) Anonymous, *Western Druggist*, **9**, 387(1887).
(2) Edel, F., *Pharm. Era*, **19**, 667(1898).
(3) Beringer, G. M., Jr., *Proc. Am. Pharm. Assoc.*, **57**, 112 (1909).
(4) Wolff, J. E., DeKay, H. G., and Jenkins, G. L., THIS JOURNAL, **36**, 407(1947).
(5) Sperandio, G. J., and DeKay, H. G., THIS JOURNAL, *Pract. Pharm. Ed.*, **10**, 572(1949).
(6) Smyth, H. F., Jr., *et al.*, *J. Ind. Hyg. Toxicol.*, **23**, 259(1941).
(7) Smyth, H. F., Jr., *et al.*, *ibid.*, **24**, 281(1942).
(8) Powers, J. L., and Crossen, G. E., "Scoville's The Art of Compounding," 7th ed., The Blakiston Company, Philadelphia, Pa, 1943.

Filter Technique as a Factor in the Stability of Thiamine Hydrochloride Injections*

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The stability of thiamine hydrochloride injection is influenced, among other factors, by the nature and preparation of the filter used in the bacteriological or polishing filtration. Of three filters studied, the Seitz type proved least desirable. The Pyrex fritted glass candle type was found superior, and the Selas 03 candle, previously ignited and then washed with hydrochloric acid and water, proved second best.

MANY VARIABLES may affect the stability of thiamine hydrochloride in parenteral solution (1, 2): the manufacturing source and particular batch of raw material; the concentration of solution; the *p*H; the presence of trace heavy metals; the bacteriostatic agent used; the nature of the ampul or vial and closure, and their cleansing; the conditions of storage such as temperature, nature of the atmosphere above the solution, and length of storage; the method of sterilization; and other manufacturing procedures.

Little has been published concerning the effect upon thiamine stability of different techniques of aseptic filtration or of obtaining filtrates of a high degree of clarity. Most widely used filtration processes involve the use of a Selas micro-porous porcelain filter candle of 02 or 03 porosity. Also employed, particularly where pyrogen elimination is desired, are the Seitz and Ertel type asbestos filters. In connection with both the Selas and the pyrogen-removal filters, an additional line filter of stoneware, alundum, or fritted glass of fine porosity is frequently used for removing stray fibers or for polishing the filtrate to a relatively high degree of clarity.

It is the purpose of the present study to compare the effects of several filter techniques, particularly with reference to the nature and preparation of the filter. Selas 03, Pyrex fritted glass ultra-fine, and Seitz filters were selected for investigation.

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PROCEDURE

Selas candles, made by sintering porcelain particles of controlled size, have largely replaced the Chamberland, Berkefeld, and Mandler candles because of the former's greater degree of mechanical strength and resistance to heat and chemicals. Selas 03 candles with glazed porcelain connecting nipples, having a diameter of 1.55 cm., a filter length of 5 cm., and maximum pore radius of 0.6 μ, were used in the experiments.

Selas candles act by particle retention rather than by adsorption and therefore should be cleaned frequently. Washing with water and chromic acid cleaning fluid and other reagents, as well as ignition methods, have been recommended for cleaning. In the present study, three methods of cleaning were selected. To avoid possible contamination of the thiamine hydrochloride solution from traces of retained reagents, it was decided to limit the washing liquids to water (distilled) and hydrochloric acid.

One method of cleaning consisted of washing the Selas candle by gentle suction with 100 cc. of hydrochloric acid U. S. P., immersing for one week in hydrochloric acid, flushing with water until free of acid, drying, and igniting in a Selas candle furnace at 540° for five hours. After cooling, the candle was flushed with 1 L. of water. In the second method, the candle was first ignited at 540° for five hours, cooled, and flushed with 1 L. of water, then with 100 cc. of hydrochloric acid, then immersed for one week in hydrochloric acid, and finally flushed free of acid with water. In the third method, the Selas candle was washed simply with 2 L. of water under slight vacuum. In each of the three methods, the integrity of the Selas candle was checked by the Bubbling Pressure Test as recommended by the manufacturers (3).

The Seitz filter used was type EK-6, containing a sterilization-type asbestos pad having a diameter of 6 cm. and a thickness of 0.2 cm. A new pad was used for the filtration of each sample of thiamine hydrochloride solution. The asbestos pad was washed with 4 L. of water before use. In the filtration setup, an additional line filter of fine sintered glass was used to remove stray asbestos fibers from the filtrate.

The Pyrex fritted glass filter, No. 35000, was an ultra-fine tubular (candle) filter with fused glass connecting nipple. The candle had a diameter of 1.55 cm. and a 5-cm. length of fritted section. The maximum pore radius was 0.6 μ. The filter was flushed under vacuum with 100 cc. of hydrochloric acid, immersed in hydrochloric acid for one week, and then flushed free of acid with water.

All the filters after cleaning were set up in filter flasks for aseptic vacuum filtration and autoclaved at 15 lb. (121.5°) for twenty minutes just before use.

The thiamine hydrochloride solutions were prepared in water for injection at a concentration of 50 mg./cc. Ampul grades of thiamine hydrochloride were obtained from three sources: Merck & Co., Inc.; Chas. Pfizer & Co., Inc.; and Hoffmann-LaRoche, Inc. The solutions were adjusted to pH 4 (glass electrode) with sodium hydroxide U. S. P. As bacteriostatic agents, a combination of 0.02% propylparaben and 0.18% methylparaben was used throughout. The solutions, after aseptic filtration through each of the differently prepared filters, were then filled into 5-cc. clear glass, washed and sterilized Kimble ampuls, and sealed under an atmosphere of nitrogen. One half of each batch was steamed at 100° for thirty minutes.

Ampuled solutions were assayed, tested for sterility, and stored in the dark at 23° ± 4° for one year. Results which indicate the effect on the color, on the formation of sediment, and on the percentage decomposition of thiamine hydrochloride are shown in Table I.

SUMMARY

There are no significant differences in percentage of thiamine decomposition when thiamine hydrochloride injections are filtered through Pyrex ultra-fine fritted glass filters or Selas 03 porcelain filters prepared by either of the three processes described, but Seitz filtration results in slightly greater thiamine loss.

However, the effects of different filter techniques upon color change and sedimentation are more marked. Amber coloration and heavy precipitation result from the Seitz filter or from the water-washed Selas candle. With respect to the ignition treatment of Selas candles, sedimentation is heavier when a candle is washed with hydrochloric acid and water and then ignited, than when these processes are reversed. Both processes, however, give less precipitation and discoloration than is the case when the Selas candle is washed only with water.

Discoloration and sedimentation are at a minimum when a hydrochloric acid- and water-washed, ultra-fine Pyrex fritted glass candle is used.

The source and particular lot of the thiamine hydrochloride is a factor which may affect its stability adversely even when filtration conditions are at optimum.

Heat treatment of ampuls of thiamine hydrochloride injection at 100° for one-half hour results in a slightly increased deterioration compared to aseptic handling, under practically all types of filter techniques studied.

Both the ultra-fine Pyrex fritted glass filter candle and the Selas 03 candle, first ignited and then washed with hydrochloric acid and water, yield solutions which meet U. S. P. XIV standards for thiamine hydrochloride injection, which permit a slight turbidity or precipitate.

DISCUSSION

In attempting to explain the somewhat superior action of the Pyrex fritted glass candle over the porcelain Selas candle in the filtration of thiamine hydrochloride solutions, the difference in composition of the two filter media is of interest. Pyrex, essentially a borosilicate glass, contains considerably less alumina and alkali oxides, no lime, and only minute traces of heavy metals, compared to porcelain in which relatively larger percentages of these are present. The term "chemical inertness" is a relative one. Even slight quantities of some of these components, extracted by solutions of low pH, may have a det-

TABLE I.—RELATIVE STABILITY OF THIAMINE HYDROCHLORIDE INJECTIONS PROCESSED BY DIFFERENT FILTER TECHNIQUES, AFTER ONE YEAR AT 23° ± 4°

	B_1 Source No. 1			B_1 Source No. 2			B_1 Source No. 3		
	Color[a]	Pptn.[b]	% B_1 Loss	Color[a]	Pptn.[b]	% B_1 Loss	Color[a]	Pptn.[b]	% B_1 Loss
Selas Candle (acid-washed and ignited)									
Aseptic	Y	2	6	Y	3	7	Y	2	5
Steamed, 100°	Y	3	7	Y	3	8	Y	3	7
Selas Candle (ignited and acid-washed)									
Aseptic	Y	1	5	Y	1	7	Y	1	6
Steamed, 100°	Y	1	6	Y	2	7	Y	1	6
Selas Candle (water-washed)									
Aseptic	A	3	7	A	4	7	A	2	6
Steamed, 100°	A	4	7	A	4	8	A	3	8
Pyrex UF Candle (acid-washed)									
Aseptic	C	0	4	Y	1	6	C	0	5
Steamed, 100°	C	1	5	Y	1	6	C	1	6
Seitz Filter (water-washed)									
Aseptic	A	4	10	A	3	10	A	3	9
Steamed, 100°	A	4	11	A	4	12	A	4	10

[a] Color is expressed as follows: C, colorless; Y, yellowish; A, amber.
[b] Degree of precipitation is expressed numerically as follows: 0, clear; 1, very faint spiral cloud formed upon rotation of upright ampul; 2, slight cloud; 3, light sediment; 4, heavy sediment.

rimental effect upon thiamine stability. While no comparable data are available, it is interesting to note that in a National Bureau of Standards study (4) it was shown that Pyrex glass, when exposed to a 5 per cent solution of sodium chloride acidified with hydrochloric acid (*p*H of about 4), showed less erosion than any other glass. It may also be noted that distilled water produced three times as much erosion on the Pyrex glass, and a neutral solution of sodium chloride produced over one hundred times as much erosion.

It is obvious that no single type of filter is suitable for the filtration of all injectable solutions. At neutral or high *p*H, or where ignition of the candle is necessary to remove certain organic precipitation impurities, the fritted glass candle may not prove as applicable as the Selas candle filter, properly prepared.

For the sensitive thiamine hydrochloride solution, however, the Pyrex fritted ultra-fine glass filter, now available in candle form, does provide a suitable filter for producing a sterile, clear solution of good stability.

With respect to commercial applicability, the flow rate of the Pyrex UF candle filter compares favorably with the Selas 03 candle. Under 30 mm. pressure, the flow rate of the Pyrex filter, of 1-inch diameter and 8-in. fritted glass length, was found to be approximately 7 L. per hour for a thiamine hydrochloride solution prefiltered through Whatman No. 4 paper. For the Selas 03 candle of the same dimensions, the flow rate under the same conditions was found to be approximately 6.5 L. per hour.

REFERENCES

(1) Taub, A., Katz, I., and Katz, M., THIS JOURNAL, **38**, 119(1949).
(2) Proceedings of the Scientific Conference of the American Pharmaceutical Manufacturers Association, 1940, p. 134.
(3) "Selas Micro-porous Porcelain Filter Media," Selas Corp. of America, Philadelphia, 1949, p. 26.
(4) Wichers, A. N., and Clabaugh, W. S., *Ind. Eng. Chem., Anal. Ed.*, **13**, 419(1941).

Preparation and Storage of Autoclaved Pectin Solutions*

By T. H. SCHULTZ, H. LOTZKAR, H. S. OWENS, and W. D. MACLAY

Solutions of autoclaved pectin, designed to be useful as a blood-plasma extender, have been clarified by centrifugation and use of bacterial filters. Filtration rates and effectiveness of several types of filters are evaluated. Storage studies showed that these solutions can be kept at least two years under refrigeration with little change. At body temperature and the *p*H of blood, the degradation of pectin into smaller molecules is relatively faster, which may be of importance in promoting elimination at a satisfactory rate and preventing harmful effects.

IN RECENT years, there has been considerable interest in development of suitable extenders for blood plasma. Pectin received attention during World War II and results of tests in about 600 clinical cases have been reported (1). From these reports, it appeared to be nonallergenic and, when autoclaved to alter its physical properties, showed no serious adverse physiological after-effects. The material is a commercial product of immediate availability and adequacy of supply.

The purpose of this paper is to report work of recent years at this Laboratory on methods of filtering and effects of storage on solutions of pectin autoclaved to an average molecular weight within the limits required for a blood-plasma extender.

METHODS AND MATERIALS

Preparation of Solutions.—The samples of pectin N. F.[1] used had the following properties: anhydrouronic acid content, 85%; methoxyl content, 10.2%; intrinsic viscosity, about 4 dl. per Gm. Solutions, 1.5% in concentration, were made with distilled water and autoclaved in 2-L. flasks at 118° for fifteen minutes. Total time, including warm-up and cooling to 100°, was one hour. After this treatment, the number average molecular weight of the pectin was about 18,000, a lower value than that for the plasma proteins. Because of the shape of the pectin molecule (2), however, this low value is required for its solutions in order to make both the viscosity and colloid osmotic pressure close in value to these properties of blood. The weight average molecular weight was probably about twice as high, or about 30,000 to 40,000, which is in the range of pectins used by other investigators (1).

Reagent-grade sodium chloride was dissolved in three of the four autoclaved pectin solutions used in the storage studies to make the salt concentration 0.90%. After clarification, the *p*H of one of these solutions was increased to 7.34 by addition of sodium orthophosphate. All but one of the filtered pectin solutions used in the storage studies were protected from contamination by microorganisms with 0.001% of phenylmercuric borate.

Centrifuging Equipment.—Preliminary clarification, in which most of the insoluble matter was removed, was done with a Sharples Super Centrifuge, with a bowl 1.75 inches in diameter, operated at 20,000 to 50,000 r. p. m.

Filtration.—Data on the bacterial filters used are given in Table I. A comprehensive discussion of the physicochemical and bacteriological principles underlying this type of filtration is given by Mudd (3).

Filtration rate measurements at 20 lb. per sq. in. and 25° ± 0.5° were made by collecting the filtrate in 500-ml. graduated cylinders and recording the time in seconds required for the volume to reach each 50-ml. mark. Fifty-milliliter cuts were taken at intervals for determination of viscosity and turbidity.

Solutions for the storage studies were filtered through a 2 x 10-inch, No. 12 Mandler candle, using suction into a 4-L. flask. The assembled unit had been autoclaved thirty minutes at 118°, with openings protected by cotton plugs.

Procedure for Cleaning Filter Candles.—The usual method of cleaning consisted of backwashing with hot tap water at 22 lb. per sq. in. for about one hour, followed by washing in 0.1 *N* hydrochloric acid, thorough rinsing with distilled water, and drying by suction for five minutes or several hours in a vacuum oven at 70°. In a few cases the candle was cleaned enzymatically[2] with Pectinol 100D[3] at *p*H 3.7 and autoclaved in addition to the regular cleaning.

Bottling and Storage.—Sterilized 100-ml. bottles were used for storage studies. After the bottles were filled and the plastic caps were wrapped with Parafilm, they were placed in constant temperature rooms at 0°, 24°, and 35° ± 0.5°. Specimens taken at the various time intervals consisted of separate bottles of solution.

Physical Measurements.—Viscosity determinations were made with 10-ml. samples in a No. 100 Ostwald-Cannon-Fenske pipette calibrated with water, in a bath at 25° ± 0.03°. The relative viscosity is the ratio of the viscosity of the solution to the viscosity of the solvent. Where salt is present,

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[1] Manufactured by California Fruit Growers Exchange, Ontario, Calif. Mention of this and other products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

[2] Berkefeld candles used in filtration of blood plasma have been advantageously cleaned with pepsin solution (4).

[3] Manufactured by Rohm and Haas Co., Philadelphia, Pa.

TABLE I.—DATA ON BACTERIAL FILTER CANDLES USED

Brand Name	Principal Material	Size, In.	Porosity No.	Bubbling Pressure, Lb./Sq. In.	Maximum Pore Diameter, Microns
Mandler	Diatomaceous earth	1 x 8	13	13	3.3
Mandler	Diatomaceous earth	1 x 8	14	14	3.0
Selas	Porcelain	5/8 x 6	01	7	6.0
Selas	Porcelain	5/8 x 6	02	25	1.7
Selas	Porcelain	1 x 8	03	35	1.2
Seitz[a]	Asbestos[a]	Dia. = 2[a]	Serum No. 1	...	...
Mandler	Diatomaceous earth	2 x 10	12	12	3.5

[a] The filtering element is a sheet instead of a candle.

the solvent is considered to be 0.90% sodium chloride solution. Osmotic pressures were determined as described earlier (2).

Turbidity determinations were made with a Lumetron photoelectric colorimeter, using the pickup attachment for measuring scattered light at right angles to the incident beam and a 590-mμ color filter. The instrument was adjusted to zero with water and balanced at 100 on the scale with barium sulfate sol as standard. The standard was prepared by mixing 25 ml. of 95% ethanol, 5 ml. of barium chloride solution (100 Gm. of the dihydrate and 50 ml. of 37% hydrochloric acid per liter), 10.0 ml. of 0.0100 *N* sulfuric acid, and distilled water to make a total of 50.0 ml. The sol was used in the colorimeter within twenty minutes after preparation. For routine tests, use was made of a secondary standard, consisting of a white tile set against the end of the pickup attachment. Turbidity readings were corrected to what they would have been if the intensity of primary light had been 100% throughout the solution and all of the scattered light directed toward the pickup photoelectric cells had actually reached them. This was necessary because the color of some of the storage samples increased. A rough idea of what the turbidity values mean is obtained from the observation that solutions with readings as high as 17 (corrected) appear practically water-clear, if viewed in a 100-ml. bottle close to the window with a dark background. A solution with a rating of 30 is slightly turbid. Sample bottles taken in the storage studies were shaken and then allowed to stand ten minutes to become free from air bubbles before filling the colorimeter sample cell.

Color intensity measurements were made with a Klett-Summerson photoelectric colorimeter, using a 40-mm. sample cell and a 420-mμ color filter. The sample was not shaken before this test. The instrument was adjusted to zero with water in the cell.

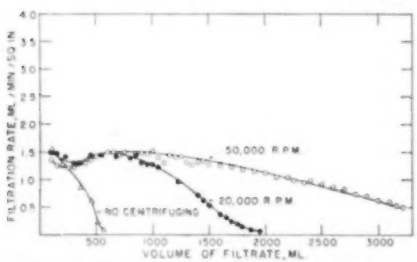

Fig. 1.—Filtration of autoclaved pectin solution. Effect of speed of centrifuging before filtering. Filter candle, 1 x 8-inch Mandler No. 14.

RESULTS

Value of Centrifuging before Filtration.—If we define the capacity of a filter candle as the volume in ml. received before the rate decreases to 0.50 ml. per minute per square inch, the capacity of a fine-grade Mandler candle was increased from 470 ml. to 1,520 and 3,200 by super-centrifuging at 20,000 and 50,000 r. p. m., respectively, as shown in Fig. 1. The three measurements were made in the order named with the same filter candle, cleaned between runs by the ordinary backwashing procedure. The improvement probably would have been even more striking if enzymatic cleaning had been used. Minima and maxima in the early part of the rate curves, as shown in Fig. 1, were obtained only when the candle had been dried at 70° instead of at room temperature.

Comparison of Types and Grades of Bacterial Filters.—Rates of filtration with filters of three types are shown in Fig. 2. The abscissae of the points for the Selas No. 02 and Seitz curves were increased to what they would have been if the filtering

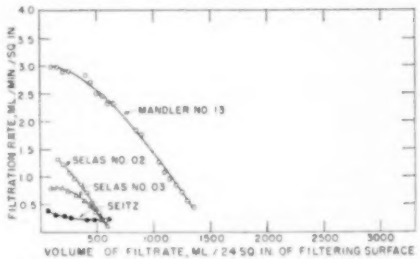

Fig. 2.—Filtration of autoclaved pectin solution, centrifuged at 20,000 r. p. m. Comparison of filters.

areas were 24 sq. in., the same as with 1 x 8-inch candles. Filtration was considerably more rapid through the Mandler candle than with filters of the other types.[4] Capacities, as defined above, were 1330, 480, and 430 ml., respectively, for Mandler

[4] Selas No. 015 candles, with a maximum pore diameter of 2.8 μ, were not available at the time of these experiments.

No. 13, Selas No. 02, and Selas No. 03 candles. The curve for the Mandler candle is representative of what could be expected for repeated use of the same candle with proper cleaning between runs. Both of the Selas candles were new. One trial was made with a Selas No. 01 candle. The rate was very fast, remaining constant at 44 ml. per minute per square inch up to a volume of 4,470 ml., but the clarity of the solution was not appreciably improved.

Repeated Use of Filter Candles.—Filtration rates in successive runs with a stock solution and with the same Mandler No. 13 candle, cleaned by the usual backwashing method between runs, are shown in Fig. 3. The capacity of the candle decreased from 2,780 ml. in the first run to 500 ml. in the fifth and sixth. After the sixth run, the candle was cleaned enzymatically. The filtration rate in the next run was improved and the capacity increased to 1,520 ml.

Effect of Filtration on Turbidity.—The turbidity of 1.5% solution of autoclaved pectin, after centrifuging at 20,000 r. p. m., usually was 40 to 50. The solution which was centrifuged at 50,000 r. p. m. was rated 29.

When the centrifuged solution was filtered at a pressure of 20 lb. per sq. in. through a Mandler No. 13 or 14 candle, the turbidity of the first 50 ml. of filtrate was very low, from 1 to 6. As filtration progressed, the turbidity increased and then remained nearly constant at about 25 during the last third of the run. The turbidity of the filtrate obtained with the Selas No. 03 candle varied from 1 on the first cut to 13 at the end. The whole filtrate obtained with the Seitz filter was rated at 10.

Effect of Filtration on Ash Content.—The silica content was reduced from 0.2 to 0.3% to about 0.025% (based on weight of dry pectin) when coarse-grade Mandler candles were used in earlier filtration experiments. However, the ash content increased considerably, in one instance from 0.7 to 2.4%. This is believed due to ion exchange reactions, cations being taken from tap water during backwashing. After a hydrochloric acid wash was added to the cleaning procedure, little, if any, increase in ash content was observed. In the present experiments, the ash content of the original pectin was 0.59%. After centrifuging at 20,000 r. p. m., it was 0.30%. Subsequent filtration with Mandler No. 14 and Selas No. 03 candles gave 0.32% and 0.24%, respectively. The greater part of the remaining ash consists of salts of calcium, aluminum, sodium, and magnesium.

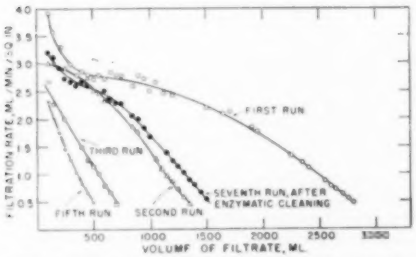

Fig. 3.—Filtration of autoclaved pectin solution, centrifuged at 20,000 r. p. m. Repeated use of filter candle, 1 x 8-inch Mandler No. 13.

TABLE II.—CHANGE IN VISCOSITY OF AUTOCLAVED PECTIN SOLUTIONS DURING STORAGE AT 0°, 24°, AND 35° C.

Storage Time, Days	—Relative Viscosity at 25° C. for Solns. Stored at: 0° C.	24° C.	35° C.
Pectin Concn. = 1.56%. NaCl Concn. = 0.90%. Natural pH.			
0	6.73	6.73	6.73
31	6.71	6.47	5.70
62	6.72	6.25	4.80
91	6.71	6.09	4.22
122	6.70	5.88	3.74
215	6.65	5.44 5.44[a]	2.49 3.01[a]
459	6.60	4.52	1.55 2.63[a]
760	6.55	3.74	1.28
Pectin Concn. = 1.54%. No salt. Natural pH.			
0	7.61	7.61	7.61
31	7.61	7.39	6.82
62	7.60	7.21	6.24
221	7.58	6.59 6.55[b]	4.37
461	7.57 7.47[b]	6.00 5.88[b]	2.58
Pectin Concn. = 1.59%. NaCl concn. = 0.90%. Initial pH = 7.34.			
0	6.0	6.0	6.0
10	5.97	5.32	4.51
22	5.95	5.10	4.29
62	5.89	4.82	3.86
216	5.76	4.59	2.91
459	5.72	4.27	2.02

[a] No preservative used.
[b] Storage is in Pyrex bottles.

Effect of Filtration on Pectin Concentration and Viscosity.—Filtration through fine-grade candles had little effect on the pectin concentration, provided filtration was stopped when the rate decreased to 0.50 ml. per minute per square inch. Relative viscosities of samples taken during a number of the runs were consistent with the relative viscosity of the unfiltered solution. However, when filtration through a Mandler candle was continued until the rate fell to 0.05 ml. per minute per square inch, the relative viscosity of the final cut was 5.35, compared with 5.87 before filtering. This effect was more striking with the Selas No. 03 candle which gave a final value of 4.37 under the same conditions. The first half of the filtrate from the run with the Seitz filter had a relative viscosity of 5.85, and the second half showed a relative viscosity of 5.49.

Storage Stability.—Changes in viscosity of autoclaved pectin solutions during storage are shown in Table II and Fig. 4. Storage was in soft-glass bottles and preservative was present in all cases except where noted to the contrary. In 0.90% sodium chloride, at natural pH,[5] the specific viscosity (relative viscosity minus one) changed only 3.1% during a period of two years at 0°. The rate increased rapidly with temperature. At 24° the decrease in specific viscosity was 51%, and at 35° it was 95% over a two-year period. During the same time

[5] The term "natural pH" refers to the unadjusted pH of the solution, which was 3.3 to 3.6. In this investigation it is not the true natural pH of pectinic acid because the pectin had been partially neutralized with ammonia by the manufacturer.

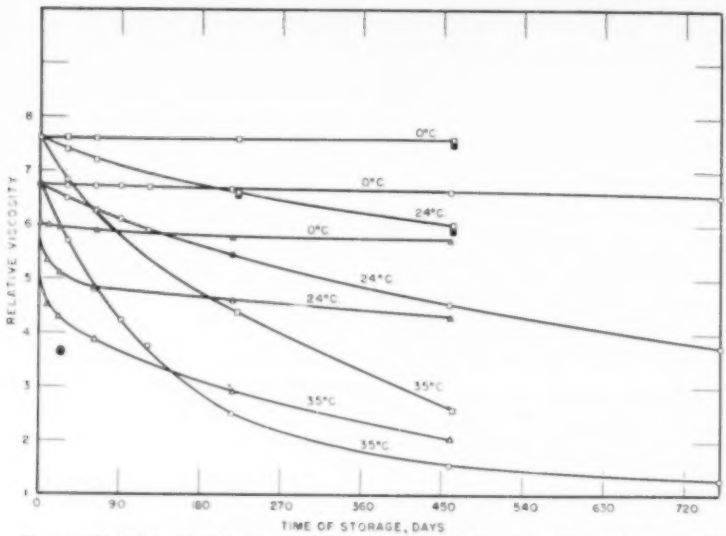

Fig. 4.—Relative viscosity of autoclaved pectin solutions *vs.* time of storage at 0°, 24°, and 35°C. ○, sodium chloride concn. = 0.90%, natural *p*H; ×, same, but without preservative; △, sodium chloride concn. = 0.90%, initial *p*H = 7.35; □, no salt, natural *p*H; ■, same but in Pyrex bottles.

at 0° the change in number average molecular weight, from osmotic pressure measurements, was less than experimental error. At 24° it decreased from about 18,000 to about 13,000. At 35° degradation was somewhat greater in the presence of phenylmercuric borate, although at 24° there was no difference in rate. The reason for this difference is not clear.

Solution which contained no salt was more stable than those containing sodium chloride, the rate of decrease in viscosity being 33–57% as fast. This appears to be due to the fact that salt lowers the *p*H of pectin solution. Solution stored in Pyrex bottles decreased in viscosity slightly more than that stored in soft-glass bottles.

Solution which was adjusted to *p*H 7.35 at the beginning showed a rapid decrease in viscosity at first, but later, after the *p*H had decreased, the change in viscosity was slower than in the corresponding solutions at natural *p*H, indicating that the optimum *p*H for stability is near 4 or 5.

Changes in *p*H.—Figure 5 shows decreases in *p*H which occurred during storage. The solution containing salt, at natural *p*H, decreased from 3.36 to 3.27 during two years at 0°. At 24° the final *p*H was 3.01 and at 35° it was 2.60. The kind of glass in the storage bottles had no measurable effect on the *p*H.

Turbidity of Storage Samples.—The effect of storage on turbidity of autoclaved pectin solutions is shown in Fig. 6. In 0.90% sodium chloride, at natural *p*H, there was no significant change in turbidity over a two-year period at 0° or 24°. All of the samples stored at 35° increased in turbidity due to the formation of a flocculent brown precipitate which appeared as sediment after seven months. There was no appreciable difference between the samples stored in Pyrex and in soft-glass bottles. Half of the samples which contained no preservative became moldy despite precautions to keep the solution sterile during bottling. At 0° it required between two and seven months for the mold to become visible.

Color Development.—Changes in color intensity during storage are shown in Fig. 7. At natural *p*H there was very little color development at 0°, the samples became light yellow at 24°, and yellowish brown at 35°. At initial *p*H 7.35 the solution became yellow immediately upon addition of the sodium phosphate solution. Then, during storage, there was a relatively rapid increase in color at first, after which there was practically no change, except at 35°.

DISCUSSION

Clarification.—Choice of the most satisfactory large-scale method for clarification of autoclaved pectin solution depends on a number of factors, such as costs of various filters, cost of cleaning filter candles, the clarity specification for the finished solution, etc., as well as upon filtration rates and capacities of the various types and grades of filters. From the data in this report it appears probable that the best method would consist of centrifugation with a high field, followed by filtration with a system having as high a capacity per unit area as is consistent with the degree of clarity required and with removal of microorganisms. If filter candles are used, filtration should be stopped before the rate falls much below 0.5 ml. per minute per square inch, since further operation gives little yield and is time consuming. At rates lower than this, the pores are so clogged that part of the pectin molecules are

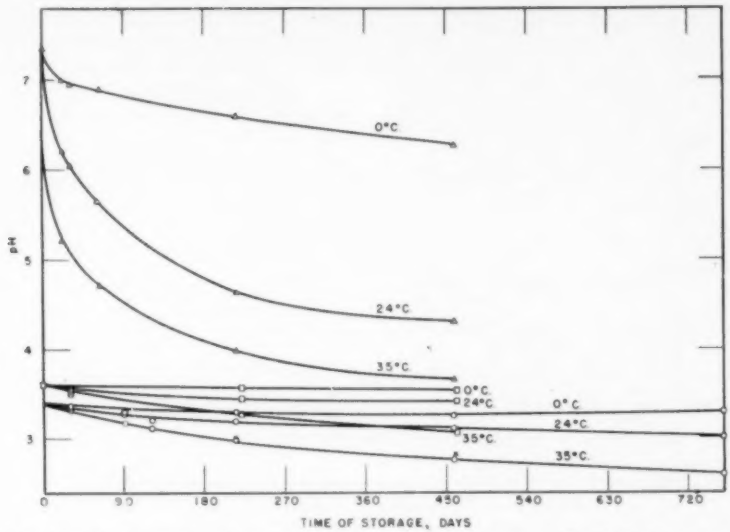

Fig. 5.—pH of autoclaved pectin solutions vs. time of storage. Symbols have same meaning as in Fig. 4.

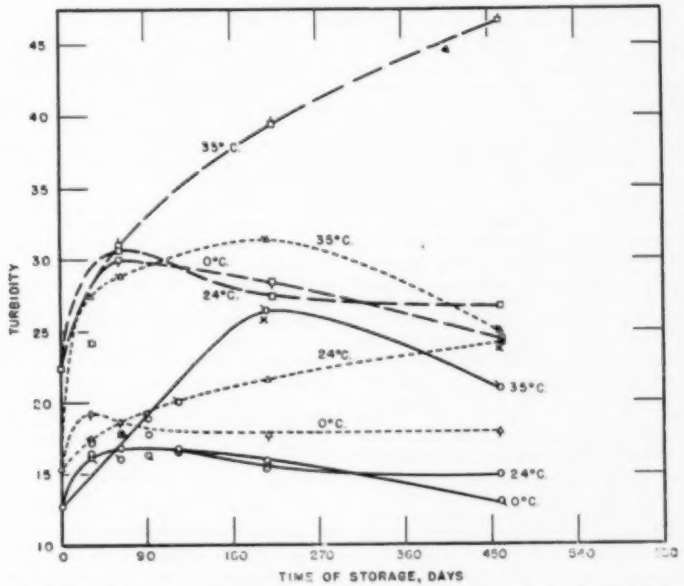

Fig. 6.—Turbidity of autoclaved pectin solutions vs. time of storage. Symbols have same meaning as in Fig. 4.

strained from the solution, judging from the observed decrease in viscosity of the filtrate.

The exact nature of the relatively large colloidal particles, which remain after super-centrifuging and cause turbidity, is not known. Autoclaved pectin molecules contribute very little, if any, to scattering of light, for the first cut of filtrate with a Selas No. 03 candle showed a turbidity reading only $^1/_{24}$ that of the unfiltered solution, although viscosity data showed no difference in pectin concentration. The increase in filtrate turbidity as filtration proceeds indicates that some of the particles are a little

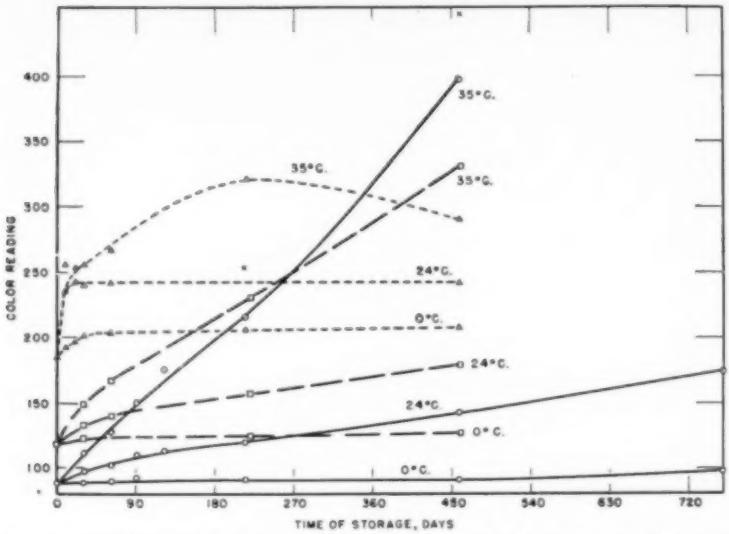

Fig. 7.—Color intensity of autoclaved pectin solutions *vs.* time of storage. Symbols have same meaning as in Fig. 4.

smaller than the pores in the candle. Their travel is impeded but they gradually work their way through. The remainder of the particles are about the same size as the pores, or larger, and therefore are strained out or retained in the filter. This material is presumed to be mainly siliceous matter, because the silica content is greatly reduced by the over-all clarification treatment. However, the candle-cleaning experiments show that a considerable part of the material which clogs the pores is subject to enzymatic action. This may be due to a small amount of pectin, acting as a binder on siliceous matter, but it appears more likely that part of the material of relatively large particle-size is a nonpectin carbohydrate. The enzymes in Pectinol hydrolyze a number of different carbohydrates. Enzymatic cleaning of filter candles would probably be economical and it is likely that the time required for cleaning could be materially reduced below that described here.

Storage.—The high stability of autoclaved pectin solution containing 0.9% sodium chloride at 0° shows the practicability of preparing solutions for intravenous infusions two years or more before use, provided they are stored under refrigeration. Relatively short storage at room temperature would not be harmful. Stability could probably be improved further by adjustment of the *p*H to about 4 or 5. If it is desired to adjust the *p*H to that of blood, it would be better to add sodium phosphate solution just before infusion, because degradation, as indicated by viscosity change, is faster at the higher *p*H. This effect may be important in breaking down pectin molecules to such size that they are slowly eliminated by the kidneys and have only transient effects, if any, on the spleen, liver, and other organs (5). In the blood stream the *p*H would be maintained above 7 and pectin degradation would be faster than indicated in Fig. 4, where the *p*H was not constant. This decrease in *p*H, shown in Fig. 5, is due to deësterification of pectin. The relationship between viscosity and molecular weight of pectin has been discussed earlier (2).

The data on viscosity and osmotic pressure of the solution in 0.90% sodium chloride and at natural *p*H have been used in a consideration of the kinetics of pectin degradation. Reaction rates for breaking of bonds between the galacturonide or other residues were calculated to be 0.15×10^{-6}, 5.3×10^{-6}, and 30×10^{-6} reciprocal days at 0°, 24°, and 35°, respectively, on the assumption that the reaction is of the first order. These rates lead to an activation energy of about 29,000 calories per mole, in agreement with the results of experiments on degradation at higher temperatures (6). This value is close to those reported for hydrolysis reactions in which glycosidic bonds are broken, confirming the belief that degradation of pectin involves breaking bonds of this type.

SUMMARY

1. Autoclaved pectin solutions, designed to be useful as a blood-plasma extender, have been clarified by centrifugation followed by filtration with bacterial filters. Rates and capacities of filters were determined. Enzymatic cleaning of filter candles was more effective than other methods tried.

2. Storage studies showed that clarified, autoclaved pectin solutions can be kept at least two years under refrigeration, with little change. Relatively short storage at room temperature is not harmful. At body temperature and at the

*p*H of blood, the degradation of pectin into smaller molecules is relatively faster, which may be of importance in promoting elimination at a satisfactory rate and preventing harmful effects.

3. The temperature coefficient of the rate of degradation at a *p*H of about 3 confirms the belief that degradation of pectin involves breaking of glycosidic bonds.

REFERENCES

(1) Joseph, G. H., "Pectin Sols for Parenteral Use," Research Department, California Fruit Growers Exchange, Ontario, Calif., 1950.
(2) Owens, H. S., Lotzkar, H., Schultz, T. H., and Maclay, W. D., *J. Am. Chem. Soc.*, **68**, 1628(1946).
(3) Mudd, S., in "Filterable Viruses," Williams and Wilkins Co., Baltimore, Md., 1928, Chapter II.
(4) Witlin, B., *Science*, **98**, 160(1943).
(5) Small, C. S., Bryant, E. F., and Palmer, G. H., *Arch. Surg.*, **60**, 575(1950).
(6) Merrill, R. C., and Weeks, M., *J. Am. Chem. Soc.*, **67**, 2244(1945).

Certain Substituted Thiobisacetamic Acids and Their Salts*

By LEWIS C. LAPPAS†, ‡ and GLENN L. JENKINS

The syntheses of the following six substituted thiobisacetamic acid derivatives and two potassium salts are described: (*a*) N-(β-benzyloxyethyl)-thiobisacetamic acid; (*b*) N-(β-benzyloxyethyl)-α,α′-thiobis-*n*-propionamic acid; (*c*) N-(β-benzyloxyethyl)-α,α′-thiobis-*n*-butyramic acid; (*d*) N-(β-benzhydryloxyethyl)-thiobisacetamic acid; (*e*) N-(β-benzhydryloxyethyl)-α,α′-thiobis-*n*-propionamic acid; (*f*) N-(β-benzhydryloxyethyl)-α,α′-thiobis-*n*-butyramic acid; (*g*) potassium N-(β-benzyloxyethyl)-thiobisacetamate; (*h*) potassium N-(β-benzyloxyethyl)-α,α′-thiobis-*n*-butyramate. The following intermediates are reported for the first time: (*a*) α,α′-thiobis-*n*-propionic anhydride; (*b*) benzyloxyethylamine; (*c*) β-(α-methylbenzyloxy)-ethylamine. Preliminary pharmacological data indicated that potassium N-(β-benzyloxyethyl)-thiobisacetamate was slightly spasmogenic and relatively nontoxic. Potassium N-(β-benzhydryloxyethyl)-thiobisacetamate did not display spasmogenic or spasmolytic activity and appeared to be nontoxic.

ANAPHYLACTIC REACTIONS have been minimized and often eliminated through the use of an antihistaminic drug which is thought to exhibit a competitive inhibitory effect against the histamine produced in allergic reactions. Of the many antihistaminic drugs produced for therapy, there is still to be found any one agent which is devoid of toxic side reactions, gives a consistent response in an individual, and has an extended duration of action.

It has been observed that the sulfur atom and the amide linkage have appeared individually in varying structural patterns of effective antihistaminic agents. It was deemed feasible to combine these groups in a common molecule, together with the ethylamine moiety which is so characteristic of many of the proved antihistaminic drugs.

This investigation consists of attempts to prepare various substituted thiobisacetamic acid derivatives. The presence of the carboxyl group in these compounds offers a means of preparing soluble salts which enhance pharmacological administration.

The substituted thiobiscarboxylic acids were synthesized by reacting the sodium salts of the α-halogenated acids with freshly prepared sodium sulfide and subsequent acidification.

The cyclization of the thiobiscarboxylic acids as the anhydrides, caused with acetyl chloride, makes possible the presence of *cis-trans* isomerism with the *trans* form existing as the dextro- or levorotatory isomer, and the *cis* form being the optically inactive or the *meso* isomer. In all instances, the mixture of isomers was used in the subsequent syntheses without attempting to isolate the individual isomers.

The aralkyl-ether-amines were prepared by reacting metallic sodium with ethanolamine and treating the formed sodium alcoholate with the α-halogenated aralkyl compound.

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The various substituted thiobisacetamic acid derivatives were synthesized by treating the corresponding thiobiscarboxylic anhydrides with the aralkyl-ether-amines under anhydrous conditions. The amic acids produced were oily to resinous in character and all of those, except N-(β-benzhydryloxyethyl)-α,α′-thiobis-*n*-butyramic acid, resisted attempts toward crystallization. Direct distillation of the oily amic acids at 8 mm., 1 mm., and 0.09 mm. resulted in decomposition.

An attempt to purify N-(β-benzyloxyethyl)-thiobisacetamic acid by preparing the ethyl ester from ethyl alcohol saturated with hydrogen chloride and subsequent distillation caused hydrolysis of the amide linkage.

The use of alcoholic potassium hydroxide for the preparation of the salts of the amic acids was inconsistent, as only two salts (Table I) could be made while the others exhibited hydrolysis of the amic acids at the amide linkage. Various attempts to prepare the salts of these amic acids with aqueous solutions of sodium carbonate and potassium carbonate produced waxy water-soluble compounds which could not be crystallized. All efforts to obtain crystalline ammonium salts from the cold ethereal solutions of the amic acids with dry ammonia gas were unsuccessful.

EXPERIMENTAL

Preparation of the Thiobiscarboxylic Acids

Preparation of Thiobisacetic Acid.—Thiobisacetic acid was synthesized according to a procedure by Loven (1) which was modified by evaporation of the acidified aqueous solution to dryness *in vacuo* (15 mm.) and extraction of the residue with ether.

Preparation of α,α′-Thiobis-*n*-propionic Acid.—Schacht (2) first reported the synthesis of α,α′-thiobis-*n*-propionic acid. The procedure used in this work was that of Loven (1) for the preparation of thiobisacetic acid with the following modification: The final acidified aqueous solution was evaporated *in vacuo* (15 mm.) until an oil layer was observed above the aqueous layer, and this mixture was subsequently extracted with ether.

Preparation of α,α′-Thiobis-*n*-butyric Acid.—Loven (3) was the first to propose a method of synthesis for α,α′-thiobis-*n*-butyric acid. The method used to prepare this acid in this work was identical to that described for the preparation of α,α′-thiobis-*n*-propionic acid.

Preparation of the Thiobiscarboxylic Anhydrides

Preparation of Thiobisacetic Anhydride.—Thiobisacetic anhydride was prepared according to the method of Anschutz and Biernaux (4) by refluxing thiobisacetic acid with an excess of acetyl chloride. The resultant anhydride was recrystallized from anhydrous chloroform in a yield of 71%. This anhydride was also obtained in a yield of 71% by direct distillation at 158–159° at 12 mm. [reported, 158° at 10 mm. (5)]; m. p. 101° [reported, 102° (4)].

Preparation of α,α′-Thiobis-*n*-propionic Anhydride.—Twenty-seven grams of dry α,α′-thiobis-*n*-propionic acid was heated at reflux temperature under anhydrous conditions with 38 cc. of acetyl chloride for a period of three hours. The resultant product was distilled *in vacuo* to remove the excess acetyl chloride and the formed acetic anhydride, and the colorless oil, distilling at 133–137° at 14 mm., was collected. The total yield was 19.8 Gm. or 81.5% of the theoretical; n_D^{25}, 1.5010.

Anal.—Calcd. for $C_8H_{10}O_3S$: N. E., 80.0. Found: N. E., 80.0.

Preparation of α,α′-Thiobis-*n*-butyric Anhydride.—The method of preparation for this acid was that of Rasanen and Jenkins (6). These workers prepared the anhydride from both the *meso* form and the racemic mixture of α,α′-thiobis-*n*-butyric acid with similar results, except that the anhydride from the *meso* form melted at 18–19° and that from the racemic form did not solidify even at −10°. The mixture of isomers of the acid was used in this experiment. The anhydride distilled at 149–150° at 15 mm. (reported, 149–151° at 15 mm.) in a yield of 96.2%; n_D^{25}, 1.4942 corresponded to that found in the literature. The oily anhydride solidified to a crystalline mass at 14–15° (reported, 18–19°).

Preparation of the Aralkyl-Ether-Amines

Preparation of Sodium Oxyethylamine.—To 61 Gm. (1 mole) of ethanolamine was added 21 Gm. (0.92 Gm. atom) of metallic sodium in small portions under anhydrous conditions. Gentle heat and stirring with a Hershberg stirrer were applied when the rate of reaction was noticeably decreased. Approximately 250 cc. of anhydrous toluene was added slowly to this mixture and the application of gentle heat continued until the evolution of hydrogen ceased. The gray mass of sodium oxyethylamine, which was insoluble in toluene, was used as such for the subsequent reactions with the desired α-halogenated compounds.

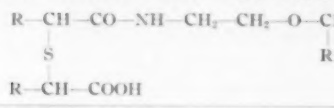
Preparation of Benzyloxyethylamine.—To the sodium oxyethylamine prepared in the previous experiment was added dropwise, under anhydrous conditions, 126 Gm. (1 mole) of benzyl chloride. Gentle heat and stirring were applied for a period of three to five hours. The mixture was allowed to cool and was filtered with suction. The precipitate was washed with several portions of anhydrous toluene and the combined washings and filtrate were distilled under vacuum. The fraction distilling at 85–125° at 1 mm. was collected. This "creeping" in the temperature range of the distillate was also observed when a second run was made at 8 mm. pressure. The latter product distilled at 109–142°. The average yield was 83 Gm. (54.3%); n_D^{25}, 1.5260.

Anal.—Calcd. for $C_9H_{13}ON$: N, 9.26%. Found: N, 9.25%.

Preparation of Benzhydryl Bromide.—The procedure followed was similar to that used by Rieveschl (7). The benzhydryl bromide, which distilled at 178–180° at 23 mm., [literature, 182° at 20 mm. (8)] was obtained in a yield of 80%.

Preparation of Benzhydryloxyethylamine.—The procedure of Sutherland, *et al.* (9), was followed for

TABLE I.—SUBSTITUTED THIOBISACETAMIC ACID DERIVATIVES[a]



R	R′	Yield, %	n_D^{25}	M. P., °C.	Formula	N Analysis, % Calcd.	N Analysis, % Found	N. E. Calcd.	N. E. Found
H	H	63.5	1.5592	...	$C_{12}H_{17}O_4NS$	4.95	4.54	283	284.9
CH_3	H	74.3	1.5427	...	$C_{13}H_{21}O_4NS$	4.5	4.26	311	303
C_2H_5	H	84.0	1.5364	...	$C_{17}H_{25}O_4NS$	4.13	3.82	339	335.2
H	C_6H_5	49.5	1.5698[b]	...	$C_{18}H_{21}O_4NS$	3.9	3.79	359	368.9
CH_3	C_6H_5	71.0	1.5630[c]	...	$C_{23}H_{23}O_4NS$	3.62	3.33	387	388
C_2H_5[d]	C_6H_5[d]	61.0	...	114[e]	$C_{23}H_{29}O_4NS$	3.38	3.39	415	416
H (K salt)[f]	H	52.4	...	204[e, g]	$C_{13}H_{16}O_4NSK$	4.36	4.49	...	...
C_2H_5 (K salt)[f]	H	53.0	...	160–162[e, g]	$C_{17}H_{24}O_4NSK$	3.71	3.88	...	...

[a] The attempts to prepare a series of the amic acids from the thiobiscarboxylic anhydrides and β-(α-methylbenzyloxy)-ethylamine, using the general procedure, yielded oily products, the analyses of which did not compare favorably for the desired compounds.
[b] n_D^{25}.
[c] $n_D^{28.5}$.
[d] Crystallized from ethereal solution in the cold (0° C.) after six days.
[e] Recrystallized from absolute alcohol.
[f] Cold alcoholic solution of the corresponding acid neutralized with cold alcoholic potassium hydroxide solution of known strength.
[g] Melted with decomposition.

the preparation of benzhydryloxyethylamine. The viscid colorless oil, distilling at 185–186° at 4 mm. (reported, 150–153° at 0.3 mm.), crystallized on cooling; and the product which was recrystallized from ether melted at 74–75° (reported, 73–74°); yield, 95.5 Gm. (42%).

Preparation of β-(α-Methylbenzyloxy)-ethylamine.—This compound was synthesized in the same manner as that previously described for the preparation of benzyloxyethylamine using one mole of α-phenylethyl bromide. The colorless oil, distilling at 105–108° at 2 mm., was collected in a yield of 40.6%; n_D^{25}, 1.5290.

Anal.—Calcd. for $C_{10}H_{15}ON$: N, 8.49%. Found: N, 8.03%.

Preparation of the Substituted Thiobisacetamic Acid Derivatives (Table I)

General Procedure.—A definite amount of the thiobiscarboxylic acid anhydride (8–12 Gm.) was placed in a 250-cc. three-neck flask equipped with reflux condenser, calcium chloride drying tube, dropping funnel, and mercury seal stirrer. To this was added, under anhydrous conditions, 50 cc. of anhydrous benzene with subsequent heating on a steam bath for five minutes. An equimolar amount of the aralkyloxyethylamine was dissolved in 50 cc. of anhydrous benzene and added dropwise to the anhydride with constant stirring. This mixture was heated at reflux temperature for a period of one-half hour. The excess benzene was removed at reduced pressure (15 mm.) and the residue was neutralized with a saturated solution of sodium carbonate until a *p*H of 9.0 was attained. The alkaline solution was diluted to five times its volume with distilled water. This mixture was then extracted with three 200-cc. portions of ether and the ether extractions were discarded. The aqueous layer was treated with activated charcoal and heated on a steam bath for five minutes. This mixture was filtered through Filter-Cel with suction until the filtrate was clear. The filtrate was acidified with a 3.5% solution of hydrochloric acid to a *p*H of 5.0. The resultant mixture was extracted with ether until the aqueous layer was clear. The aqueous layer was discarded and the ether layer was dried over Drierite. The ether was removed by distillation and the remaining oil was redissolved in acetone and treated with activated charcoal and heated over a steam bath for five minutes. This mixture was then filtered and the Drierite was washed with anhydrous acetone with subsequent evaporation of the acetone *in vacuo* (15 mm.). The final product, which was oily to resinous in character, was subjected to analysis.

SUMMARY

1. The syntheses of six substituted thiobisacetamic acid derivatives and two potassium salts are described.

2. Preliminary pharmacological tests indicated that potassium N-(β-benzyloxyethyl)-thiobisacetamate was slightly spasmogenic and relatively nontoxic. Potassium N-(β-benzhydryloxyethyl)-thiobisacetamate did not display spasmogenic or spasmolytic activity and appeared to be nontoxic.

REFERENCES

(1) Loven, J. M., *Ber.*, **27**, 3059(1894).
(2) Schacht, C., *Ann. Chem. Pharm.*, **129**, 1(1864).
(3) Loven, J. M., *J. prakt. Chem.*, **33**, 102(1886).
(4) Anschutz, R., and Biernaux, F., *Ann.*, **273**, 68(1893).
(5) Richter, V., translation by Alicott, E. N., "Organic Chemistry," Vol. I, Nordeman Publishing Co., Inc., New York City, 1934, p. 430.
(6) Rasanen, P. R., and Jenkins, G. L., THIS JOURNAL, **38**, 599(1949).
(7) Rieveschl, G., Jr., U. S. pat. 2,421,714, June 3, 1947; through *Chem. Abstr.*, **41**, 5550(1947).
(8) Bui-Hoi, N. P., *Ann.*, **556**, 1(1944).
(9) Sutherland, L. H., Eberlin, S. L., Forsythe, J. D., Halverstadt, I. F., Vaugn, J. R., and Clapp, R. P., *J. Org Chem.*, **14**, 235(1949).

A Simple Method for the Determination of Organic Bound Iodine*

By BENNIE ZAK† and ALBERT J. BOYLE‡,§

A rapid method for the determination of organic bound iodine is described using chloric acid as the oxidizing digestion reagent. The iodine concentration which may be determined with analytical accuracy ranges from a fraction of a milligram to 100 mg. The method permits the simultaneous estimation of organic bound iodine in several samples since a minimum of care is indicated in the digestion process. No special apparatus is required other than that found in the ordinary laboratory. The procedure makes possible the analysis of 20 samples per day per analyst.

A MODIFICATION of the chloric acid procedure for the submicro determination of protein bound iodine(1) has been used succesfully in this laboratory for the estimation of micro, semimicro and macro amounts of organic bound iodine, where the weight of the iodine ranged between a fraction of a milligram and 100 mg. In order to prove the accuracy of the method, the titrimetry was checked by means of polarography and spectrophotometry using standard solutions to prepare calibration curves so that absolute values could be found for the iodine in thyroid extract as well as for pure organic compounds. A second check is readily apparent then from the use of the latter, for the theoretical amounts of iodine present were calculated to show how successful recoveries have been. This is apparent from perusal of Tables I through V.

PROCEDURE

Several procedures for micro and macro amounts of iodine are described in the literature (2–7). Among the most widely used methods, either as it is written or in some modification thereof, is the one described in the United States Pharmacopeia (8), which involves an alkaline fusion of the iodine-containing organic sample in a muffle furnace, extraction of the residual melt with distilled water, and boiling of the extract obtained with elemental bromine. The latter converts iodine to iodate, which is the desirable form for quantitative estimation. After destruction of the excess bromine and careful adjustment of the *p*H, six equivalents of iodine are liberated and then titrated with a standard sodium thiosulfate solution using starch to indicate the end point.

The micro method of Shahrokh (9) involves the use of a mixture of sodium chlorate, perchloric acid, and dihydrogen sodium phosphate as the digestion reagent. The organic matter containing the iodine is put in a Kjeldahl flask with several milliliters of the digestion reagent, carefully heated until the characteristic fumes of perchloric acid appear, and then cooled. After the addition of sodium hydroxide and neutralization of the excess base with hydrochloric acid using thymol blue as an indicator, chlorine in carbon tetrachloride is added to decolorize the indicator. It is then evaporated by boiling until 15 ml. remains. A crystal of phenol is added before the iodine is liberated with excess potassium iodide to circumvent any possible interference by manganese if it were present in the organic material. Titration of the liberated iodine is then carried out with 0.001 *N* sodium thiosulfate solution using one drop of 1% starch as the end-point indicator.

The method described in this communication requires a 150-ml. beaker in place of a Kjeldahl flask and chloric acid digestion reagent. The latter is added to the iodine-containing organic compound and the mixture evaporated to fumes of perchloric acid. The iodine present in this solution as iodate may be estimated then by titrimetry, spectrophotometry, or polarography. Chloric acid rather than sodium chlorate is used to avoid high salt concentrations of sodium perchlorate in the final digest.

Reagents.—All chemicals used were reagent grade.

Chloric Acid.—Weigh 500 Gm. of potassium chlorate into a 4,000-ml. beaker, add 900 ml. of distilled water, and heat the mixture until solution is effected. Add 375 ml. of 72% perchloric acid with constant stirring throughout the addition. Potassium perchlorate will be present as a precipitate at this time. Cool and place the covered beaker in the freezing compartment of a refrigerator for two to three hours, and afterward decant the supernatant liquid through Whatman 41H paper. The final solution represents approximately a 28% solution of chloric acid.

Standard Iodine Solution (100.0 γ/ml.).—Weigh out 168.5 mg. of dried analytical reagent potassium iodate and dilute to 1,000 ml.

Sodium Thiosulfate (0.1 *N*).—Dissolve 24.85 Gm. of the pure crystals in water which has previously been sterilized by boiling. Add 1 ml. of 0.1 *N* sodium hydroxide, cool, and dilute to 1,000 ml. Standardize indirectly against potassium iodate (10).

Sodium Thiosulfate (0.01 *N*).—Dissolve 2.50 Gm. of the pure crystals in hot water and treat as above.

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Sodium Thiosulfate (0.001 *N*).—Dissolve 0.250 Gm. of the pure crystals in hot water and treat as previously described.

The Cadmium Iodide-Linear Starch Reagent (11).

EXPERIMENTAL

To a 150-ml. beaker containing a weighed sample of an organic iodine compound add 10–25 ml. of the chloric acid digestion reagent and evaporate at the low temperature of the hot plate to fumes of perchloric acid.

Titrimetric.—Cool and dilute to about 50 ml. with distilled water. Neutralize the solution to phenolphthalein with 20% sodium hydroxide solution. Add 1 ml. of 1:1 phosphoric acid, 1 ml. of 1:10 hydrochloric acid, and 1 ml. of the cadmium iodide-linear starch reagent. Titrate with 0.1 *N*, 0.01 *N*, or 0.001 *N* sodium thiosulfate, depending upon the approximate concentration of iodine present to the disappearance of color of the iodo-starch blue complex. Typical results of titrimetric determination of iodine in thyroid extract and of organic bound iodine are shown in Tables I and II.

Polarographic.—Dilute the digested sample in a 100-ml. volumetric flask with 0.2 *N* sodium hydroxide containing 1.5 Gm. of sodium sulfite per 1,000 ml. The reducing agent is present here to remove dissolved oxygen so that no interference will be encountered due to the fact that oxygen is reduced in two steps at the dropping mercury cathode at a voltage span which includes iodate within it. An amount of 5–6 ml. of the solution is transferred to the electrolysis cell and a polarogram is drawn with the aid of a Sargent XXI polarograph. The concentration of iodine is then determined from a calibration curve on which the log of the diffusion current and the log of the concentration are ordinate and abscissa, respectively. The half-wave potential for this system was found to be −1.13 volts *versus* the calomel electrode. Typical results of polarographic determination of organic bound iodine are shown in Table IV.

Spectrophotometric.—Dilute the digested sample to the desired volume, depending on the size of the sample and the iodine content. To a 2-ml. aliquot add 1 ml. of 1% potassium iodide solution. The color due to liberated iodine is measured on a Beckman DU spectrophotometer at either 288 or 353 mμ. The lower peak at 353 mμ is preferable with the more concentrated solutions. Typical results of spectrophotometric determination of organic bound iodine are shown in Table III. The three methods are compared in Table V.

TABLE I.—DATA FOR TITRIMETRIC DETERMINATION OF IODINE IN THYROID EXTRACT

Weight, Mg.	I_2 Present,[a] γ	I_2 Added, γ	I_2 Found, γ	Deviation,[c] γ
64.8	126.4	0.0	123.1	− 3.3
64.8	126.4	0.0	136.1	+ 9.7
64.8	126.4	0.0	136.1	+ 9.7
64.8	126.4	0.0	129.6	+ 3.2
64.8	126.4	0.0	129.6	+ 3.2
64.8	126.4	0.0	116.6	− 9.8
64.8	126.4	0.0	123.1	− 3.3
64.8	126.4	0.0	123.1	− 3.3
64.8	126.4	0.0	123.1	− 3.3
64.8	126.4	0.0	123.1	− 3.3
64.8	126.4	0.0	129.6	+ 3.2
37.5	73.3	0.0	75.0	+ 1.7
65.3	127.4	0.0	137.1	+ 9.7
50.8	99.4	0.0	111.9	+12.5
64.8	126.4	0.0	123.1	− 3.3
121.6	236.5	118.8	361.8	+ 5.5
59.0	138.8	118.8	242.7	−14.9
53.6	124.1	118.8	255.8	+12.9
0.0	0.0	118.8	113.0	− 5.8
0.0	0.0	118.8	118.1	− 0.7
0.0	0.0	118.8	122.6	+ 3.8
0.0	0.2 Gm. KBr[b]	118.8	116.3	− 2.5
0.0	0.2 Gm. KBr	118.8	116.3	− 2.5
0.0	0.2 Gm. KBr	118.8	122.3	+ 3.5

[a] The amount present is based on the arithmetical mean calculated from the values obtained on 12 separate samples.
[b] This was carried out to check the possibility of bromide interference.
[c] Mean deviation (14) of a single observation is equal to ±5.6 γ.

TABLE II.—DATA FOR TITRIMETRIC DETERMINATION OF ORGANIC BOUND IODINE

Sample	Weight, Mg.	I_2 Present, Mg.	I_2 Found, Mg.
I—C_6H_4.NH.$COCH_3$	35.7	17.35	17.51
I—C_6H_4.NH.$COCH_3$	15.3	7.44	7.47
I—C_6H_4.NH.$COCH_3$	15.2	7.39	7.47
I—C_6H_4.NH.$COCH_3$	14.9	7.24	7.27
I—C_6H_4.NH.$COCH_3$	36.8	17.91	17.88
I—C_6H_4.NH.$COCH_3$	164.7	77.3	80.00
I—C_6H_4.NH.$COCH_3$	76.2	34.5	37.0
I—C_6H_4.NH.$COCH_3$	33.2	16.2	16.14
I—C_6H_4.NH.$COCH_3$	127.7	60.59	62.06
CH_2:I_2	51.9	49.4	49.6
CH_2:I_2	62.4	59.4	59.7
CH_2:I_2	61.4	58.4	58.5
$C_6H_{12}C_3H_3I$	20.8	8.57	8.56
$C_6H_{12}N_4C_3H_3I$	21.3	8.77	8.75
$C_6H_{10}N_4C_3H_3I$	20.0	8.24	8.17

DISCUSSION

The chloric acid procedure for micro and macro quantities of organic bound iodine is precisely the same. The fuming process is very brief and the loss of iodine appears to be negligible. While this laboratory has employed a specially prepared starch solution in the titration procedure, ordinary starch is satisfactory for amounts of iodine in the ranges shown. The phosphoric acid is added to complex any iron per cent which would give rise to high results. If it is known that iron is absent, the phosphoric acid may be omitted.

One postulated mechanism for the decomposition of chloric acid is as follows (page 262):

TABLE III.—DATA FOR SPECTROPHOTOMETRIC[a] DETERMINATION OF ORGANIC BOUND IODINE

Sample	Weight, Mg.	I_2 Present, Mg.	I_2 Found, Mg.
Standard 1	..	2.98	..
Standard 2	..	8.91	..
Standard 3	..	17.81	..
Standard 4	..	23.74	..
$C_6H_{12}N_4C_3H_3I$	8.3	3.44	3.42
I—$C_6H_4NHCOCH_3$	38.8	18.86	18.79
I—$C_6H_4NHCOCH_3$	15.0	7.29	7.35

[a] Wave length was 288 mμ.

TABLE IV.—DATA FOR POLAROGRAPHIC[a] DETERMINATION OF ORGANIC BOUND IODINE

Sample	I_2 Present, Mg.	Id, ma.	Ht., mm.	Sensitivity	I_2 Found, Mg.
Standard I	0.2374	0.231	77.0	0.003	...
Standard II	2.98	0.81	81.0	0.01	...
Standard III	8.91	2.40	121.5	0.02	...
Standard IV	23.74	4.68	117.0	0.04	...
Standard V	23.74	6.72	112.0	0.06	...
$I—C_6H_4 \cdot NH \cdot COCH_3$	18.86	5.16	129.0	0.04	19.27
$I—C_6H_4 \cdot NH \cdot COCH_3$	7.29	2.01	67.0	0.03	7.31
$C_4H_{12}N_4C_5H_4I$	3.33	0.98	98.0	0.01	3.41
Thyroid (129.6 mg.)	0.2528	0.243	81.0	0.003	0.2497
Thyroid (129.6 mg.)	0.2528	0.246	82.0	0.003	0.2498

[a] $E_{1/2}$ was −1.13 v.

TABLE V.—COMPARISON OF TITRIMETRIC, SPECTROPHOTOMETRIC, AND POLAROGRAPHIC DETERMINATIONS OF ORGANIC BOUND IODINE

Sample	Weight, Mg.	I_2 Present, Mg.	I_2 Found, Mg.		
			Titr.	Polar.	Spect.
Thyroid extract	64.8	0.1264	0.1300	0.1260	...
Thyroid extract	129.6	0.2528	0.2460	0.2490	...
$C_6H_{12}N_4C_5H_4I$	8.1	3.34	3.32	3.41	3.40
$I—C_6H_4NHCOCH_3$	39.0	18.86	18.78	19.27	18.76
$I—C_6H_4NHCOCH_3$	15.0	7.29	7.25	7.30	7.35

$$2HClO_3 \rightarrow HClO_4 + HClO_2$$
$$HClO_2 + HClO_3 \rightarrow 2ClO_2 + H_2O$$
$$2ClO_2 \rightarrow Cl_2 + 2O_2$$
$$3HClO_3 \rightarrow HClO_4 + Cl_2 + 2O_2 + H_2O$$

According to Luther's rule (12, 13), chloric acid must therefore be a better oxidizing agent than perchloric acid, which is probably the reason its action is so excellent for our purposes.

SUMMARY

The method proposed in this manuscript enables the worker to accurately carry out as many as 20 sample determinations in a day, since very little care need be given to the individual sample. No extensive preparations are required and the number of manipulations per sample has been reduced to a minimum.

REFERENCES

(1) Zak, B., *et al.*, to be published.
(2) Shahrokh, B. K., and Chesbro, R. M., *Anal. Chem.*, **21,** 1003(1949).
(3) Custer, J. J., and Natelson, S., *ibid.*, **21,** 1005(1949).
(4) Chaney, A. L., *Ind. Eng. Chem., Anal. Ed.*, **10,** 326 (1938).
(5) Thompson, J. J., and Oakdale, U. O., *J. Am. Chem. Soc.*, **52,** 1195(1930).
(6) Willard, H. H., and Thompson, J. J., *ibid.*, **52,** 1893 (1930).
(7) McClendon, J. F., and Forester, G. L., *J. Biol. Chem.*, **154,** 619(1944).
(8) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950, p. 627.
(9) Shahrokh, B. K., *J. Biol. Chem.*, **147,** 109(1943); *ibid.*, **154,** 517(1944).
(10) Willard, H. H., and Furman, N. H., "Elementary Quantitative Analysis," D. Van Nostrand Company, Inc., New York, 1947, pp. 266–267.
(11) Lambert, J. L., *Anal. Chem.*, **23,** 1247(1951); *ibid.*, **23,** 1251(1951).
(12) Ephraim, F., "Inorganic Chemistry," Interscience Publishers, Inc., New York, 1947, pp. 376–377.
(13) Luther, R., *Z. physik. Chem.*, **36,** 1385(1901).
(14) Jasper, J. J., "Laboratory Methods of Physical Chemistry," Houghton Mifflin Co., Cambridge, Mass., 1938, pp. 4–5.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Methoxinine
Glucamine
Dihydroquercetin
Choline stearate
Cystamine sulfoxide
3-Methylquinoline
Uvitonic acid
Triisopropyl phosphate
4,4′-Dipiperidine
1,8-Dichloronaphthalene
Mannonic acid
Piperine
Oxythiamine
Sulfosuccinic acid
1-Thiosorbitol
Ellagic acid
Dihydroconiferyl alcohol
Diiodoricinoleic acid
2,7,8,3′,4′-Pentahydroxyflavan
2-Hydroxy-6-methylterephthalic acid

The Arginase-Alkaloid Relationship in *Datura tatula* L.*,†

By WILLARD C. FULLER‡ and MELVIN R. GIBSON§

A method for the quantitative determination of arginase in plant material is presented. The alkaloid and arginase content of *Datura tatula* L. was determined for various parts of the plant at various ages. The quantitative alkaloid-enzyme relationship tends to support the theory that the first step in the biosynthesis of the tropinone alkaloids is the hydrolytic action of arginase on plant protein molecules containing arginine.

EVIDENCES in the literature which can be used to relate alkaloid biosynthesis to enzymically catalyzed reactions are meager. However, the fact remains that not only the ability to synthesize a given alkaloid but also the actual rate at which it is produced is controlled by hereditary means (1, 2). Thus Dawson (3) claims it is reasonable to assume that some kind of gene-enzyme-reaction relationship occurs in the formation of alkaloids.

True and Stockberger (4) reported on the relationship of alkaloids, latex, and oxidases in *Papaver*. James (5) has reported the occurrence of the enzyme arginase in members of the *Solanaceae* and has suggested that there may be a relationship between the enzyme and the alkaloids. Cromwell (6, 7) has proposed the theory that arginase acts upon plant protein and makes use of this compound through several intermediate steps to form nor-hyoscyamine or nor-atropine.

The central problems of alkaloid biosynthesis are, of course, the characterization of synthetic mechanisms and the discovery of relationships, if any, between these mechanisms and other physical and chemical systems within the organism. This investigation is concerned with the alkaloid-enzyme relationship in a selected species of drug plant, *Datura tatula* L.

The procedure adopted in this investigation was to determine quantitatively the arginase activity of the plants under observation, in the different parts (roots, leaves, stems) at different ages, and to determine the alkaloid content in the same parts at the same ages. The problem was resolved into three main divisions: propagation of the experimental plants, assay for enzyme activity, and assay for alkaloid content.

MATERIALS AND METHODS

Authentic plants of *Datura tatula* L. were grown from seeds which had been previously harvested from an experimental plot of the School of Pharmacy, the State College of Washington. Plants were grown in Palouse silt loam during the study and collections were made of roots, stems and heavy petioles, and leaves as follows: collections of plant material were made on Thursday morning of each week before 8:00 a. m. The plants selected for harvest were chosen by systematic selection throughout the plot without regard to size, apparent vigor, or other visible characteristics. Each plant was dug in such a manner that the root system would remain as nearly intact as possible. After a sufficient number of plants had been harvested, the crop was divided into halves. One-half was prepared for the enzyme assay, and the other half was prepared for the alkaloid assay. The part of the crop to be used for the enzyme assay was washed free of any adhering soil and allowed to dry just long enough to evaporate any surface moisture. These washed and dried plants were then separated into roots, stems and heavy petioles, and leaves. Each part was then sealed and frozen in an airtight, plastic, freezer bag of the polyethylene glycol type. The material was maintained in this state until ready for use.

The portion of the crop to be used for the determination of alkaloid content was similarly divided into roots, stems and heavy petioles, and leaves. Each of these parts was then dried in a laboratory oven at a constant temperature of 55–60° for two days (8). The dried specimens were ground to a coarse powder in a Wiley drug mill in order to prepare them for assay, and then they were further ground to a fine powder (80 mesh or finer) in a Raymond hammer mill. This powder was then subjected to the method of assay described under the section Alkaloid Assay.

The first collection of samples was taken when the seedlings were approximately six inches high and seven weeks old. Subsequent investigation by the enzyme assay showed that plants collected at an earlier age would perhaps reveal more complete data. Therefore another crop of plants was started later in the growing season. These plants were treated in exactly the same manner as those previously described, except that they were collected

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† This paper is based on a portion of a thesis submitted to the Faculty of the Graduate School of the State College of Washington by Willard C. Fuller in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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at an earlier age, the first collection being made when the plants were four weeks old. Collections were taken each successive week until the seventh week, when all plants were severely damaged by a killing frost. The collections of the first crop were continued from the seventh week through the fourteenth week, at which time the killing frost terminated their usefulness. The results of the first and second crops were treated statistically without designation as to source-crop. This procedure resulted in duplicate results for the seventh week: one was the result of the last collection of the late crop, and the other was the result of the first collection of the early crop.

The size of the younger plants obtained in the second crop precluded their division into roots, stems and petioles, and leaves. They were divided into just two parts: roots, stems and petioles comprised one part, and leaves comprised the other.

Several measurements were taken at the height of the growing season, and it was noted that some plants reached a height of seventy-four inches with leaves as large as nine and one-half inches in breadth and stems more than three inches in diameter at the base. The large, trumpet-shaped, lavender flowers reached a size of more than five inches in length.

ENZYME ASSAY

A number of methods have been employed to measure arginase activity. The method proposed by Van Slyke and Archibald (9) was found to be a satisfactory means for determining arginase activity in plant juice extracts and therefore was selected as the basis for analysis during this investigation. This method is essentially the incubation of the enzyme solution with a substrate of a definite concentration of arginine hydrochloride under specified conditions. The urea produced is then measured photometrically by a method first proposed by Archibald (10).

Preparation of the Enzyme Solution.—The collected samples which had been frozen, as described under the section Materials and Methods, were removed from the freezer and allowed to thaw. The tissues were then squeezed in a hydraulic press to remove as much of the plant juice as possible. These expressed juices were immediately put into containers and frozen again until needed for assay. When a sample was to be assayed, the frozen juice was thawed until it was melted but only just to completion; it was then centrifuged. This latter step was considered necessary, because it was found that sedimentation and precipitation were two factors which helped to lead to unsatisfactory results. An empirical method was instituted, and its use was the only alternative discovered in which the error due to nonuniform particle size of the sediment and precipitate, unavoidably present in the juice, could be eliminated. This method consisted of centrifuging the freshly thawed juice and using only the clear, supernatant liquid for measurement.

Reagents Used.—The arginine solution (0.1 *M*, pH 9.5) was prepared by dissolving 2.1067 Gm. of *l*-arginine hydrochloride (Merck) in 11.54 cc. of 1 *N* sodium hydroxide solution and then making up to 100 cc. with Sorensen's glycocoll-sodium hydroxide buffer solution (11).

The cobalt solution was 0.35% cobalt nitrate, reagent grade, in distilled water. The cobalt ions serve as activators for arginase activity and inhibitors for urease activity in the reaction mixture.

The metaphosphoric acid solution was 24% metaphosphoric acid, reagent grade, in distilled water. This solution was filtered through a sintered-glass funnel to remove the cloudiness which appeared.

The α-isonitrosopropiophenone solution was prepared by dissolving 3 Gm. of the chemical in sufficient alcohol (U. S. P.) to make 100 cc.

The sulfuric-phosphoric acid mixture was prepared by adding 90 cc. of concentrated sulfuric acid, reagent grade, and 270 cc. of syrupy phosphoric acid (U. S. P.) to 600 cc. of distilled water. When cool, the volume was adjusted to one liter and kept tightly stoppered with a ground-glass stopper.

A stock, standard solution of urea (4 mM) was prepared by dissolving 24.0 mg. of urea in a sufficient amount of distilled water to make 100 cc. This solution was used the same day it was prepared.

The working-standard urea solution (0.4 mM) was prepared by diluting one volume of the stock urea solution to ten volumes with the sulfuric-phosphoric acid mixture on the day the working standard was to be used.

Procedure.—A quantity (0.1 cc.) of the cobalt solution was pipetted into each 20-cc. test tube. One cubic centimeter of the supernatant juice (which had just been diluted with buffer solution) was added to each test tube. The dilution was so made that the resultant solution was within optimum transmittancy readability. Following this, 1 cc. of substrate solution (previously brought to 40° in a constant temperature bath) was added to each test tube and thoroughly mixed. These tubes were then stoppered, effectively controlling evaporation which was found to be negligible, even when the mixture was incubated at this temperature for the period of time indicated. The mixture was then allowed to incubate for forty-eight hours in a constant-temperature bath of 40°. At the end of the incubation period, the reaction was stopped by the addition to each test tube of 2 cc. of metaphosphoric acid solution.

An aliquot (2 cc.) of this acid-enzyme-substrate mixture was then made up to 10 cc. with sulfuric-phosphoric acid mixture. To this 10-cc. sample, 0.5 cc. of the 3% solution of α-isonitrosopropiophenone was added and immediately mixed. This mixture was stoppered with a cork containing air-vent grooves and placed in a lightproof boiling water bath and allowed to remain for one hour. At the end of the hour, the tubes were removed to test-tube racks and immediately covered with a double thickness of heavy black cloth to exclude any light which would tend to fade the photo-labile color produced. The per cent transmittancy was then read on a Beckman DU spectrophotometer at a wave length of 540 $m\mu$ against the reagent blank as a standard. The reagent blank was prepared by adding the metaphosphoric acid to the enzyme solution before adding the substrate, and this control was subsequently treated in the same manner as the samples. The results of the enzyme assays are given in Table I and are shown graphically in Figs. 1, 2, and 3. The unit of enzyme activity was defined as the number of micromoles of urea produced by 1 cc. of juice in one hour under the specified conditions.

ALKALOID ASSAY

The method for the estimation of total alkaloids in belladonna and stramonium as presented by Roberts and James (12) was believed to be the most practical for use in the present investigation. Certain modifications, however, were necessary for the photometric measurement with a Beckman spectrophotometer. The benzene extraction yielded too much chlorophyll for subsequent use of the Vitali-Morin reaction. A column of powdered sucrose was used to remove a large quantity of the chlorophyll. Quantitative tests were made to determine the procedure for washing the column free of all alkaloids exposed to the adsorption process. The modified process follows.

Method of Assay.—The dried plant material prepared as described under the section on Materials and Methods was used. An accurately weighed sample (about 1 Gm.) was placed in a small beaker, and five or six drops of 10% ammonium hydroxide was added and stirred until the powder was evenly moistened. Benzene, reagent grade, was added up to 5 or 10 cc., and the mixture was brought to a boil over a water bath. The mixture was washed at once into a small glass percolator and tightly packed. The percolator apparatus was devised from a chromatographic tube with a ground-glass, removable, perforated plate at the lower end. When in place, the perforated plate was covered with pure absorbent cotton before the material to be extracted was introduced. After the moistened material was firmly packed, it was extracted with further quantities of benzene until exhausted.

The column for the removal of the chlorophyll was prepared by firmly packing a 19-mm. chromatographic tube with commercial powdered sucrose to a height of 12 cm. The benzene percolate was poured into a reservoir above the packed sucrose column, and the container was washed with a little more pure benzene. When the original benzene extract had just disappeared from the top of the column, another 70 cc. of pure benzene was added to wash the column. A slight greenish-yellow color was washed through into the eluate, but upon subsequent dilution, it became negligible.

The eluate was then made up to 100 cc. in a volumetric flask with benzene. An aliquot (2 cc.) of this solution was then evaporated just to dryness in a small, porcelain, evaporating dish over a water bath. The residue was taken up in exactly 0.2 cc. of fuming nitric acid, reagent grade, and again evaporated to dryness. This residue was then dissolved in a little acetone, reagent grade, and transferred quantitatively to a 10-cc. volumetric flask. When cool, this solution was made up exactly to 10 cc., and 0.1 cc. of a fresh 3% solution of potassium hydroxide in methyl alcohol (not more than two weeks old) was added. The contents of the flask were thoroughly mixed. Exactly five minutes after mixing, the color produced was measured in a Beckman DU spectrophotometer at 540 mμ against a reagent blank.

The reagent blank was prepared in exactly the same manner as previously described for the samples except that no alcoholic potash was added to produce the purple color. The quantity of alkaloid, calculated as hyoscyamine, was determined from a prepared, absorption-concentration curve.

Results of the work on alkaloid content are given in Table II and shown graphically in Figs. 1, 2, and 3. A unit of alkaloid content was defined as the number of milligrams of alkaloid (calculated as *l*-hyoscyamine) per gram of dried powdered drug.

DISCUSSION

Examination of the graphs showing the relationship of alkaloid content and enzyme activity at different ages in corresponding parts of the experimental plants gives rise to a number of observations. There is a simultaneous "leveling-off" of the alkaloid content for all parts of the plant near the tenth week and continuing on until the fourteenth week, beyond which time no further tests were made. Beginning at the seventh week, there is a sharp increase in alkaloid content each successive week extending through the tenth week. The alkaloid content of the roots exceeds that of the stems until the eighth week, when it increases sharply with age. Leaves show a greater increase in enzyme content after the seventh week, whereas the stems and roots show a less rapid rise. The enzyme content of the stems becomes nearly constant about the eleventh week, whereas that of the roots and leaves does not show a tendency to stabilize until the thirteenth week. The curves representing alkaloid-content fluctuation in roots, stems, and leaves nearly correlate each other and essentially differ only in magnitude. There seems to be a correlation of the curves representing the enzyme content in leaves and roots, but not in stems.

TABLE I.—UNITS OF ENZYME ACTIVITY IN VARIOUS PARTS OF PLANTS AT VARIOUS AGES[a]

Age in Weeks	Roots[a]	Stems[a]	Leaves[a]
5	0.151	...	0.097
6	0.137	...	0.033
7	0.072	...	0.101
7	0.203	0.071	0.203
8	0.338	0.286	1.691
9	1.832	0.659	3.179
10	2.198	1.136	6.147
11	3.845	2.639	9.757
12	5.544	2.727	16.774
13	8.361	2.697	20.698
14	9.329	3.387	21.542

[a] Averages of three determinations reported and used for plotting on graphs.

TABLE II.—UNITS OF ALKALOID CONTENT IN VARIOUS PARTS OF PLANTS AT VARIOUS AGES[a]

Age in Weeks	Roots[a]	Stems[a]	Leaves[a]
5	0.155	...	0.368
6	0.341	...	0.549
7	0.612	...	1.062
7	0.664	0.442	1.113
8	0.923	0.904	1.713
9	1.160	1.291	1.984
10	1.357	1.611	2.147
11	1.489	1.727	2.252
12	1.522	1.806	2.140
13	1.513	1.763	2.146
14	1.580	1.812	2.230

[a] Averages of four determinations reported and used for plotting on graphs.

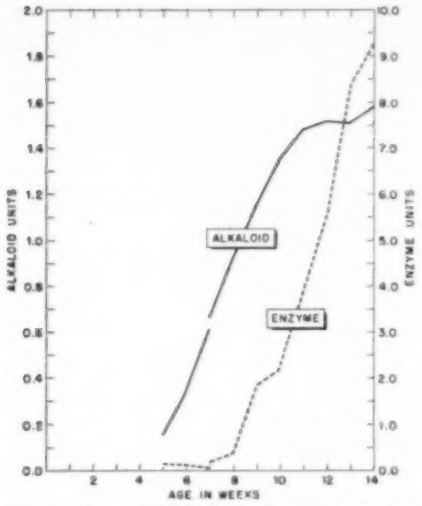


Fig. 1.—Enzyme-alkaloid relationship at various ages in roots.

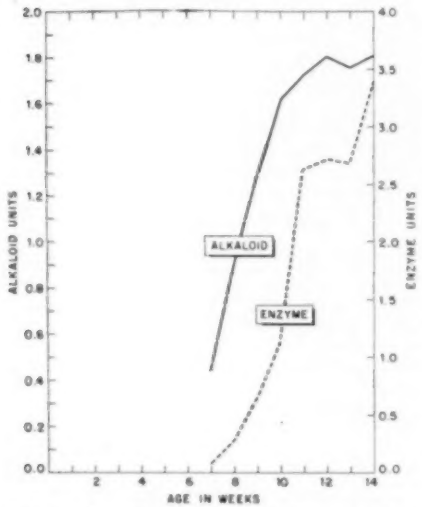


Fig. 2.—Enzyme-alkaloid relationship at various ages in stems.

The most significant observations are those which can lead to the establishment of a relationship between the alkaloid content and the arginase activity. The curve for enzyme activity in leaves is similar to the alkaloid curve for leaves in its general trend. A similar relationship may be observed between the curves representing roots. The difference between the curves is found chiefly in the fact that the enzyme curves seem to level off at a later age than those for the alkaloids. The most significant observation is that the alkaloid content increases most

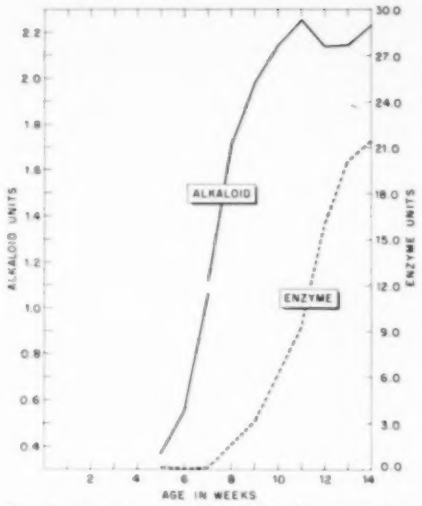


Fig. 3.—Enzyme-alkaloid relationship at various ages in leaves.

at the time the enzyme activity begins to increase tremendously in all parts.

CONCLUSIONS

It may be concluded on the basis of the foregoing observations that the enzyme arginase may play a definite role in the biosynthesis of the tropinone alkaloids of *Datura tatula* L., since an increase in enzyme activity appears at approximately the same time that sharp increases in alkaloid content are noted.

On the basis of the evidence presented, it may be concluded that there is a definite relationship between alkaloid content and arginase activity in *Datura tatula* L. This relationship tends to support the theory that the first step in the biosynthesis of the tropinone alkaloids is the hydrolytic action of arginase on plant protein molecules containing arginine.

REFERENCES

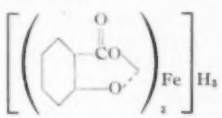
(1) Kostoff, D., "Cytogenetics of the Genus *Nicotiana*," State's Printing Office, Soña, 1941–1943, through Dawson, R., *Advances in Enzymol.*, **8,** 203(1948).
(2) Smith, H. H., and Smith, C. R., *J. Agr. Research*, **65,** 347(1942).
(3) Dawson, R., *Advances in Enzymol.*, **8,** 203(1948).
(4) True, R. H., and Stockberger, W. W., *Am. J. Botany*, **3,** 1(1916).
(5) James, W. O., "Oxford Medicinal Plants Scheme," Annual Report, 1945, p. 9.
(6) Cromwell, B. T., *Biochem. J.*, **37,** 722(1923).
(7) Cromwell, B. T., *ibid.*, **37,** 717(1942).
(8) Rubin, M., and Harris, L. E., THIS JOURNAL, **29,** 477(1950).
(9) Van Slyke, D. D., and Archibald, R. M., *J. Biol. Chem.*, **165,** 293(1946).
(10) Archibald, R. M., *ibid.*, **157,** 507(1945).
(11) Clark, W. M., "The Determination of Hydrogen Ions," Williams and Wilkins Co., Baltimore, 1925, p. 11.
(12) Roberts, M., and James, W. O., *Quart. J. Pharm. Pharmacol.*, **20,** 1(1947).

The Colorimetric Determination of Salicylates*

By R. E. PANKRATZ and F. J. BANDELIN

The iron-salicylate color reaction is utilized for the colorimetric determination of salicylate in various salicylate compounds and in pharmaceutical preparations. Optimum conditions for the development of the iron salicylate color are determined and relationships between molecular weights and absorbancies of various salicylates are established. The method is rapid and data indicate it to be reproducible within one per cent. Methods are given for various salts and esters of salicylic acid as well as for some common pharmaceutical mixtures containing salicylates.

THE WELL-KNOWN iron-salicylate reaction has long been known and variously utilized for the detection and estimation of salicylates. Although the exact composition of the violet-colored inner complex of ferric salts with salicylic acid is not known, it has been postulated to have the following structure (1).



Numerous methods using several iron salts as reagents have been published (2–4) and various degrees of accuracy have been obtained with the reaction (5, 6), but no systematic or comprehensive study of the optimum conditions for the reaction or its reproducibility has appeared, nor has any considerable mention of the determination of the esters of salicylic acid been made. Although the reaction has been used as a limit test for salicylic acid in acetylsalicylic acid in the U. S. P. (7), little application of the reaction for the general or routine colorimetric determination of salicylates in pharmaceutical products has been made. Because of the simplicity of the reaction, the ease of color development and the apparent stability of the color, it offers considerable promise as a method for the pharmaceutical control laboratory. With this in mind, an investigation of the optimum conditions for the reaction, its accuracy and reproducibility in the determination of the various salts and esters of salicylic acid commonly used in medicine was made.

EXPERIMENTAL

Reagents

(*a*) Ferric nitrate reagent; 1.0 Gm. of $Fe(NO_3)_3 \cdot 9H_2O$ dissolved in 100 ml. of 1.0% HNO_3.

(*b*) Potassium hydroxide, reagent grade.

(*c*) Ethanol, 95%.

(*d*) Standard solution of salicylic acid: dissolve 0.5 Gm. of salicylic acid in 15 ml. of ethanol and dilute to exactly 100 ml. Pipette accurately exactly 5 ml. of this solution into a 1-L. volumetric flask and dilute to volume with distilled water. Each milliliter of this solution contains 25 μg. of salicylic acid.

Determination of Optimum Conditions

The absorbancy of ferric salicylate solutions was determined between 400 and 600 mμ. The maximum absorption occurs at 525 mμ. Since the color of the ferric salicylate complex is dependent upon *p*H, the color was developed at varying *p*H levels from 1.0 to 9.0 at 1-*p*H unit increments using Sorensens glycine, hydrochloric acid, and sodium hydroxide buffer solutions. It was found that the maximum color develops at a *p*H of about 3.5 and persists to about *p*H 8.0, where the precipitation of ferric hydroxide begins. The most favorable range was found to be from *p*H 4.0 to 6.0. Solutions of *p*H values of 6.5 and higher exhibited a marked tendency to fade rapidly. In actual practice the use of a buffer is unnecessary except where a large excess of basic material is present. The great dilution of the sample usually brings the *p*H into the desired range with the use of the reagents alone.

Preparation of a Standard Curve

Aliquot portions of the standard salicylic acid solution ranging from 5 to 45 ml. in 5-ml. increments were pipetted into each of nine 50-ml. volumetric flasks. Five milliliters of ferric nitrate reagent was added to each and all were then made to volume by the addition of distilled water. The absorbancy of each of these solutions was determined on a Coleman model 14 spectrophotometer at 525 mμ. By plotting the concentration of salicylic acid against absorbancy on normal graph paper, a straight line was evolved which was in conformity with Beer's law. The slope of this line expressed as μg./ml. over absorbancy was 71.43.

Determination of Salicylic Acid in Salicylates

Sodium Salicylate.—A portion of 0.5 Gm. was accurately weighed and transferred into a 200-ml. volumetric flask. It was dissolved in a small quantity of water and sufficient water added to bring up to volume. An aliquot of 10 ml. of this solution was diluted to exactly 1 L. Aliquots ranging from 5 to 45 ml. in 5-ml. increments were transferred to each of nine 50-ml. volumetric flasks and the color developed by adding 5 ml. of ferric nitrate reagent and adjusting the volume to exactly 50 ml. The absorbancies were determined and plotted as above. The slope in terms of micrograms of sodium salicylate per ml. over the absorbancy was 83.03.

Salicylsalicylic Acid.—A portion of 0.25 Gm. was accurately weighed and transferred to a 200-ml.

* Received October 29, 1951, from the Control Laboratory, Flint, Eaton and Co., Decatur, Ill.

volumetric flask. Ethanol (25 ml.) is added to dissolve the compound, followed by 25 ml. of freshly prepared 10% KOH solution. The mixture was allowed to stand, lightly stoppered on a steam bath for two hours. It was then cooled, 5 ml. of concentrated HCl was added, and sufficient distilled water to make up to volume. Ten milliliters of this solution was diluted to exactly 500 ml. and aliquots of this solution ranging from 5 ml. to 45 ml., in 5-ml. increments, were transferred to 50-ml. volumetric flasks and the color developed by the addition of 5 ml. of ferric nitrate reagent. The readings were plotted as previously and the slope in terms of micrograms of salicylsalicylic acid per ml. over absorbancy was found to be 66.67.

Succinylsalicylic Acid.—A portion of 0.25 Gm. was accurately weighed and transferred to a 250-ml. volumetric flask. This was hydrolyzed, neutralized, and diluted as under Salicylsalicylic Acid, and a 10-ml. aliquot was diluted to 500 ml. and aliquots from 5 to 45 ml. as previously described were used to develop color, which was measured, and concentrations plotted as previously. The slope expressed as micrograms of succinylsalicylic acid per ml. over absorbancy was found to be 92.31.

Acetylsalicylic Acid.—A portion of 0.75 Gm. was accurately weighed and transferred to a 200-ml. volumetric flask and hydrolyzed, neutralized, and diluted as directed above. Ten milliliters of this solution was accurately diluted to 500 ml., and aliquots of from 5 to 45 ml. in 5-ml. increments were transferred to 50-ml. volumetric flasks and the color developed by the addition of 5 ml. of ferric nitrate reagent. The readings were plotted as previously and the slope in terms of micrograms of acetylsalicylic acid per ml. over absorbancy was found to be 93.17.

Phenyl Salicylate.—A portion of 0.4 Gm. was accurately weighed and transferred to a 200-ml. volumetric flask and hydrolyzed, neutralized, and diluted as directed above. An aliquot of 10 ml. was accurately diluted to 500 ml., and 5 to 45 ml. aliquots in 5-ml. increments were pipetted into 50-ml. volumetric flasks and the color developed by the addition of 5 ml. of ferric nitrate reagent. The readings were plotted as previously and the slope in terms of micrograms of phenyl salicylate per ml. over absorbancy was found to be 110.77.

Methyl Salicylate.—A portion of 0.5 Gm. of the liquid is accurately weighed into a 200-ml. volumetric flask. To this are added in succession, 25 ml. ethanol, 25 ml. water, and 2.5 Gm. of potassium hydroxide. This was allowed to stand on a steam bath for two hours, cooled, diluted to volume with water, and mixed. Then a 10-ml. aliquot was pipetted into a 1-L. volumetric flask and diluted to volume with water. Aliquots of this solution ranging from 5 to 45 ml. in 5-ml. aliquots were transferred to 50-ml. volumetric flasks and the color developed by the addition of 5 ml. of ferric nitrate reagent. The readings were plotted as previously and the slope in terms of micrograms of methyl salicylate per ml. over absorbancy was found to be 79.13.

Calculations

Having determined the slope of the salicylic acid line, and knowing the per cent of salicylic acid in salicylates, it should be possible to calculate the slopes of the various salicylates by multiplying the slope of salicylic acid by the inverse function of the respective percentages (Table I and Fig. 1).

TABLE I

Salicylate	Mol. Wt.	Salicylic Acid, %	Slope	
			Calcd.	Found
Salicylic acid	138.05	100	...	71.43
Sodium salicylate	160.04	86.26	82.81	83.03
Salicylsalicylic acid	258.08	106.98	66.78	66.67
Succinyl salicylate	358.12	77.11	92.62	92.31
Acetylsalicylic acid	180.06	76.67	93.17	93.17
Phenyl salicylate	214.08	64.49	110.80	110.77
Methyl salicylate	152.14	90.74	78.73	79.13

With the slope of the acid curve established it became possible to determine the salicylic acid in a sample by multiplying the absorbancy against the value for the slope with proper calculations for the dilution.

By the same procedure it became possible to calculate the percentage of salicylate by multiplying the slope of the salicylate under consideration by the absorbancy.

It was also possible to calculate the percentage of salicylate compound by dividing the slope of salicylic acid by the grams per gram of salicylic acid in the salicylate and multiplying by the absorbancy.

$$\text{Absorbancy} \times \frac{71.43}{\text{Gm./Gm. salicylic acid}} = \text{Gm./ml. salicylate}$$

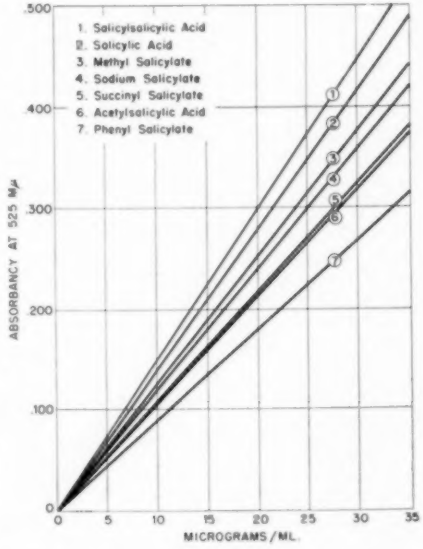


Figure 1

Or if one prefers to determine the absorbancy of a standard simultaneously while determining the Gm./Gm. salicylate, one needs a salicylic acid standard solution.

$$\text{Gm./ml. salicylic acid} \times \frac{\text{Absorbancy/Gm./Gm. salicylic acid}}{\text{Absorbancy standard}} = \text{Gm./ml. salicylate compound}$$

To establish the reproducibility of this method, a series of determinations were made using phenyl salicylate. Phenyl salicylate was selected because, as an ester of salicylic acid, it required the additional hydrolysis procedure not required by salts, thus introducing greater possibility of error either through incomplete hydrolysis, interference in color development by the phenol liberated by hydrolysis, or through possible destruction or loss of the salicylic acid.

The procedure used was that previously set forth for the determination of phenyl salicylate. Results of these determinations are given in Table II.

From these determinations it is apparent that the reproducibility of the method is within ±1% of theoretical.

DETERMINATION OF SALICYLIC ACID

Ampuls

Sodium Salicylate.—Five milliliters of solution containing 2.0 Gm. of sodium salicylate was accurately diluted to 250 ml. and 5 ml. of this solution was further diluted to 100 ml. Of this final dilution, 5-ml. aliquots were pipetted into 50-ml. volumetric flasks and the color developed as directed under Sodium Salicylate. Results are given in Table III.

TABLE II

Absorbancy at 525 mμ	μg./Ml.	Theoretical, %
0.182	20.16	100.80
0.180	19.94	99.70
0.181	20.05	100.25
0.181	20.05	100.25
0.181	20.05	100.25
0.180	19.94	99.70
0.182	20.16	100.80
0.181	20.05	100.25
0.181	20.05	100.25
0.181	20.05	100.25
0.181	20.05	100.25
0.181	20.05	100.25
0.182	20.16	100.80
0.181	20.05	100.25
0.182	20.16	100.80
0.181	20.05	100.25
0.181	20.05	100.25
0.181	20.05	100.25
0.182	20.16	100.80
0.183	20.27	101.35
0.182	20.16	100.80
0.181	20.05	100.25
0.181	20.05	100.25
0.182	20.16	100.80
0.181	20.05	100.25
0.183	20.27	101.35
0.182	20.16	100.80
0.181	20.05	100.25
0.182	20.16	100.80
0.181	20.05	100.25

TABLE III

μg./Ml. Found	Theoretical, %
19.94	99.70
19.94	99.70
19.94	99.70
19.94	99.70
19.94	99.70
19.94	99.70
20.02	100.11
20.10	100.53
19.85	99.23
19.94	99.70

Elixir Sodium Salicylate N. F.

Ten milliliters was diluted to exactly 1 L., and 10 ml. of this solution was further diluted to 100 ml. Aliquots of 10 ml. of the final dilution were pipetted into 50-ml. volumetric flasks and the color developed as before. Results are given in Table IV.

TABLE IV

μg./Ml. Found	Theoretical, %
17.20	100.10
17.03	100.18
17.03	100.18
17.03	100.18
17.20	100.10
17.20	100.10
17.11	100.67
17.03	100.18
17.11	100.67
17.11	100.67
17.03	100.18
17.03	100.18
17.11	100.67
17.03	100.18
17.03	100.18

Theobromine Sodium Salicylate Tablets

Grind 20 tablets to a fine powder and weigh the equivalent of 0.3 Gm. of salicylic acid into a 500-ml. volumetric flask. Dissolve in water and dilute to volume. Transfer 20 ml. into another 500-ml. volumetric flask and again dilute to volume. Transfer 15 ml. to a 50-ml. volumetric flask and add 5 ml. of ferric nitrate reagent; dilute to volume and determine the absorbancy at 525 mμ. Consistent and uniform results of the same magnitude as found in the determination of other salicylates were obtained.

This method may be conveniently applied to the determination of acetylsalicylic acid in APC (aspirin, phenacetin, and caffeine) tablets by extracting and hydrolyzing as directed under Salicylsalicylic Acid, or it may be used on the bicarbonate extract in Holt's (8) method of separation of the APC ingredients.

SUMMARY

The violet color of the ferric salicylate complex is applied to the colorimetric determination of salicylates in pharmaceutical compounds and preparations. The maximum spectral absorption occurs at 525 mμ and solutions of the compound conform to Beer's law. Methods are

given for the determination of salts and esters. The latter require alkaline hydrolysis to liberate salicylic acid. *p*H is a factor in the development and stability of the color, the most favorable range being from *p*H 4.0 to 6.0. Generally, *p*H adjustment is not required since the great dilutions usually necessary in the determination of salicylates in pharmaceutical compounds usually bring the solution within the optimum range. Aliquots containing from 10 to 25 μg. of salicylic acid per ml. produce an absorbancy well suited for colorimetric determination on a Coleman 14 spectrophotometer. Reproducibility within ±1.0 per cent is attained. The method may be readily and conveniently applied to the determination of salicylate in mixtures such as theobromine sodium salicylate and in APC tablets.

REFERENCES

(1) Feigl, F., "Specific and Special Reactions for Use in Qualitative Analysis," Elsevier Publishing Co., Inc., New York, N. Y., 1940.
(2) Heesterman, J. E., *Chem. Weekblad*, **32**, 463(1935).
(3) Fonss Bech, P., *Dansk. Tids. Farm.*, **9**, 289(1935).
(4) Norris, R. F., *et al.*, *Am. J. Clin. Path.*, **15**, 196(1945).
(5) Illari, G., *Ann. chim. applicata*, **28**, 524(1938).
(6) Blanca, A. M., *Rev. asoc. bioquim. argentina*, **16**, 17 (1949).
(7) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950.
(8) Holt, K. E., *Bull. Natl. Formulary Comm.*, **13**, 121 (1945).

A Polarographic Study of Some *Veratrum viride* Alkaloids*,†

By E. J. WALASZEK and A. PIRCIO

The results of a study of the polarographic behavior of jervine, veratramine, and veratrosine dissolved in a mixture of alcohol and a suitable buffer solution are reported. The method described was designed for later application to a study of the metabolism of the *Veratrum viride* alkaloids, but it demonstrates that polarographic analysis may be utilized satisfactorily in alkaloidal determination if the pure alkaloids are present in uncontaminated solutions. The *Veratrum viride* alkaloids investigated yielded characteristic reduction waves in at least two stages in the *p*H range studied.

A DETAILED polarographic study of alkaloids had not been reported in the literature until the work of Kirkpatrick (1). His investigations have clearly established that the method of polarographic analysis may be utilized in alkaloidal analysis. It was decided, therefore, to employ the polarographic technique in the study of the metabolism of the *Veratrum viride* alkaloids, which is being carried out at the present time in our laboratory. However, before attempting to make use of this technique for the development of a method of determining these particular alkaloids in biological tissue and fluid, it was deemed advisable to study their polarographic behavior in pure solution.

The present paper is concerned with the polarographic examination of the alkaloids, jervine, veratramine, and veratrosine.

* Received September 27, 1951, from the Department of Pharmacology, University of Chicago, Chicago, Ill.
† This work was done under a contract between the Atomic Energy Commission and the University of Chicago, and in part under a grant from the Medical Life Insurance Research Fund.

EXPERIMENTAL

Apparatus, Materials, and Method

A Sargent Model XII polarograph, equipped with a conventional dropping mercury cathode assembly, was used for the polarographic measurements. The cell arrangement employed was that described by Lingane and Laitinen (2). All the work was done at room temperature. The capillary was a 7.1 cm. length of marine barometer tubing and had the following characteristics: $m = 1.963$ mg. per second and $t = 2.36$ sec., with the capillary dropping into the supporting electrolyte at open circuit; the head of mercury was 81 cm. Oxygen was removed from all solutions polarographed by passing purified nitrogen for fifteen minutes. A trace of methyl red (0.1 ml. of 0.1% methyl red T. S. per 10 ml.) was added to the solutions to eliminate the maxima. Polarograms were taken at 1/20 sensitivity unless otherwise indicated. All measurements were made with respect to a mercury anode.

Half-wave potentials were measured in the usual manner, with all values being calculated with respect to the saturated calomel electrode.

The alkaloids were isolated from a commercial sample of ground, dried roots of *Veratrum viride*[1] by

[1] Kindly supplied by Irwin, Neisler and Co., Decatur, Ill.

the method of Craig and Jacobs (3). The purified alkaloids gave the following melting points:

Jervine, 240–241° (uncorr.)
Veratramine, 208–209 (uncorr.)
Veratrosine, softened at 234° and melted at 242° (uncorr.)

The buffer solutions used were prepared by mixing solutions of 0.1 *M* citric acid and 0.2 *M* disodium phosphate. The *p*H of all buffer solutions was checked with a Beckman Model G *p*H meter. Polarograms of the buffers showed no appreciable amount of reducible material before buffer breakdown. The solutions which were polarographed were prepared in the following manner: a known amount of the electroreducible alkaloid was dissolved in 0.7 ml. of ethyl alcohol. Nine milliliters of the buffer was added to this solution and the entire mixture diluted to 10.0 ml.

Results and Discussion

The polarographic data are given in Table I. The alkaloids, jervine, veratramine, and veratrosine, were found to be reducible over the entire *p*H range examined.

Current-voltage curves of these alkaloids over the *p*H range 2.2 to 5.2 have been constructed (Figs. 1, 2, and 3). In strongly acid solution, jervine yields one wave (Fig. 1, curve *I*) which is surmounted by a maximum. As the *p*H of the solution is increased, two waves are formed (Fig. 1, curve *II*) and wave *A* is displaced to a more negative potential. As the *p*H is raised to 4.2, and finally 5.2, the potential of

TABLE I.—EFFECT OF *p*H ON $E_{0.5}$ AND i_d OF JERVINE, VERATRAMINE, AND VERATROSINE

	*p*H	No. of Waves	Concn., mM	$E_{0.5}$ V.	i_d, μa.
Jervine	2.2	1	1	−1.27	2.48
	3.2	2	1	−1.29	0.40
				−1.48	2.50
	4.2	2	1	−1.24	0.20
				−1.46	2.68
	5.2	2	1	−0.88	0.20
				−1.49	2.68
Veratramine	2.2	1	1	−1.21	1.92
	3.2	3	1	−0.79	0.16
				−1.23	0.57
				−1.40	1.08
	4.2	3	1	−0.87	0.22
				−1.25	0.25
				−1.44	1.34
	5.2	1	1	−1.43	1.74
Veratrosine	2.2	1	1	−1.24	1.65
	3.2	2	1	−0.78	0.20
				−1.31	1.60
	4.2	2	1	−0.84	0.19
				−1.36	1.60
	5.2	1	1	−1.39	1.66

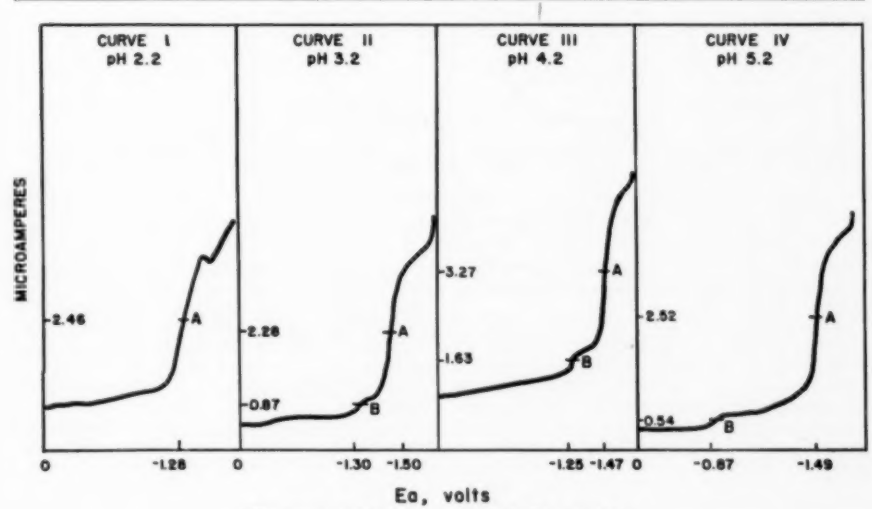


Fig. 1.—1 × 10^{-3} *M* Jervine in buffer solutions.

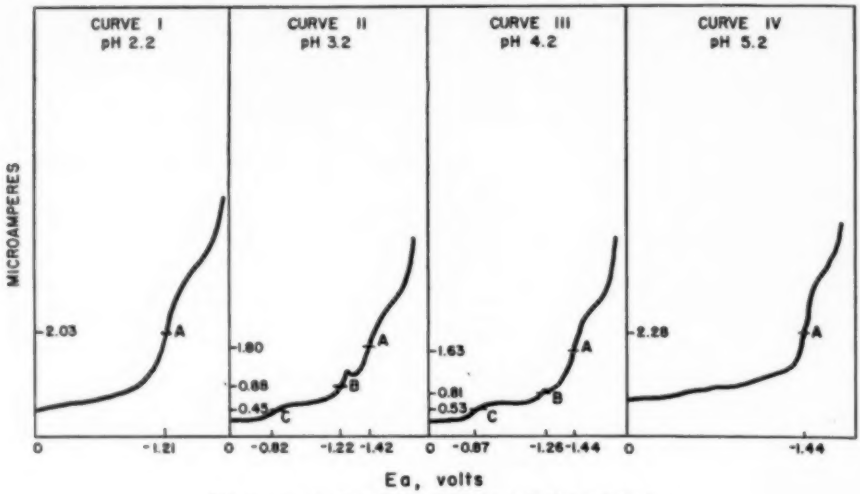


Fig. 2.—1.0×10^{-3} *M* Veratramine in buffer solutions.

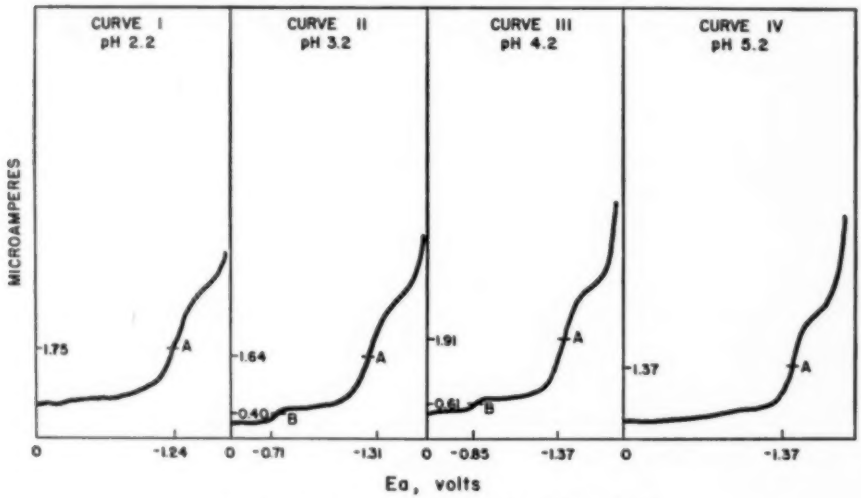


Fig. 3.—1.0×10^{-3} *M* Veratrosine in buffer solutions.

wave *A* remains practically constant while that of wave *B* is displaced to a more positive potential (Fig. 1, curves *III* and *IV*).

In the case of veratramine in strongly acid solution there occurs just one wave (Fig. 2, curve *I*). When the *p*H of the solution is increased, wave *A* is displaced to a more negative potential, and at the same time it diminishes in height with the appearance of two other smaller waves, *B* and *C* (Fig. 2, curve *II*). Wave *B* is surmounted by a maximum. At *p*H 4.2 three waves are still found (Fig. 2, curve *III*), but as the *p*H is increased to *p*H 5.2, waves *B* and *C* vanish completely (Fig. 2, curve *IV*) so that just wave *A* is present.

In strongly acid solution, as in the case of the first two alkaloids discussed, veratrosine yields one wave (Fig. 3, curve *I*). With increase of *p*H, wave *A* is preceded by a much smaller wave, *B* (Fig. 3, curves *II* and *III*) and at the same time wave *A* is displaced to a more negative potential. At *p*H 5.2, wave *B* disappears with only wave *A* appearing (Fig. 3, curve *IV*).

In all three cases the main wave (*A*) immediately precedes the rapid rise in current due to the decomposition of the supporting electrolyte.

Since $E_{1/2}$ varies with *p*H, hydrogen ions are involved in the electrode reaction. The fact that more than one wave appears in the *p*H range 3.2 to

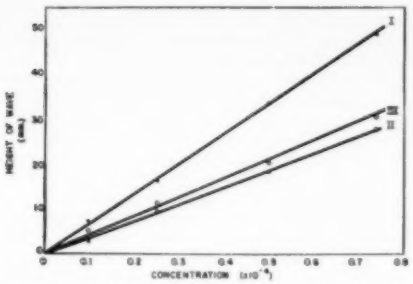


Fig. 4.—Calibration curves for pure jervine (I), veratramine(II), and veratrosine(III) at a sensitivity of 1:2.

4.2 indicates that at least two reactions are occurring in the reduction of these alkaloids. The group responsible for these reduction waves is believed to be the carbonyl group conjugated to a double bond which has been postulated to be a part of the main nucleus of the veratrum alkaloids (4, 5).

For quantitative work, reduction waves were studied at *p*H 4.5. The appropriate concentration ranges and height of waves are given in Fig. 4. These data show that a quantitative analysis of these alkaloids can be had when they are present in pure solution.

SUMMARY

1. Jervine, veratramine, and veratrosine yield characteristic reduction waves over the *p*H range studied.

2. The reduction of these alkaloids proceeds in at least two stages. In the case of jervine and veratrosine, one or two waves are observed according to *p*H of solution, while one or three waves are observed with veratramine.

3. Quantitative determination of each alkaloid can be attained polarographically when each alkaloid is present in pure solution.

REFERENCES

(1) Kirkpatrick, H. F., *Quart. J. Pharm. Pharmacol.*, **18,** 245(1945); *ibid.*, **19,** 8, 127, 526(1946); *ibid.*, **20,** 87(1947).
(2) Lingane, J. J., and Laitinen, H. A., *Ind. Eng. Chem., Anal. Ed.*, **11,** 504(1939).
(3) Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **155,** 565(1944); *ibid.*, **160,** 555(1945).
(4) Jacobs, W. A., and Sato, Y., *J. Biol. Chem.*, **175,** 57 (1948); *ibid.*, **181,** 55(1949).
(5) Freid, J., Wintersteiner, O., Moore, M., Iselin, B. M., and Klingsberg, A., *J. Am. Chem. Soc.*, **73,** 2970(1951).

Synthesis of Mersalyl Analogs*,†

By WILLIAM O. FOYE, HEMANT M. KOTAK‡, and JOHN J. HEFFERREN

The synthesis of a series of new organo-mercurial diuretics analogous to mersalyl is described. The differences in structure include the presence of other aromatic rings than benzene, and sulfonamide rather than carbonamide linkages. The compounds produced some diuretic activity, but a comparison with commercial compounds is difficult because of solubility problems.

THE INTRODUCTION of mersalyl, or Salyrgan, as an effective diuretic by Brunn (1) in 1924 has led to the synthesis of a variety of other types of mercurial diuretics but not to a great deal of analogous structures. A series of mercurated alkyl amides of dibasic phenylene acids has been reported by Tabern (2) however, as well as some mercurated pyridine carbonamides by Hartmann (3). Robbins and Chen (4) have also recently reported the testing of a large number of compounds of which the most promising was the mersalyl analog bearing a hydroxyethoxy group in place of methoxy.

It appeared desirable to prepare compounds as possible diuretics which contain less potent toxophores than the benzene nucleus, and accordingly, several thiophene, as well as naphthalene, analogs of mersalyl have been prepared. The inclusion of sulfonamide rather than carbonamide linkages also seemed desirable and convenient.

Since the fact has been established that mercurials having the configuration R—Hg^+OH^- are severely necrotizing, it has been generally found that compounds of the structure R—Hg—N— and R—Hg—S— have much less irritant action and toxicity. Because the local irritant action of the organo-mercurials appears proportional to the reactivity of the mercury, the compounds prepared are of the R—Hg—C≡ type,

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† Abstracted from the thesis presented to the Graduate School of the University of Wisconsin by Hemant M. Kotak, in partial fulfillment of the requirements for the degree of Master of Science, January, 1951.
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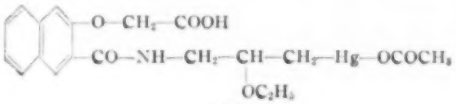
which might be expected to show less irritant action, but also lower therapeutic activity.

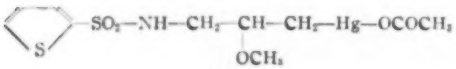
Since no detailed directions for the synthesis of mersalyl were found in the literature, it was first necessary to work out a procedure for its preparation.

PROCEDURE

Due to the low yields usually experienced on making acid chlorides of hydroxy acids, the allyl amide of salicylic acid was prepared by reacting allyl isothiocyanate with the free acid. The oxyacetic acid group was readily substituted using chloroacetic acid, and the mercuration ran smoothly using mercuric acetate, but the oily product was crystallized only after prolonged scratching under distilled water. The mersalyl analog of 2-hydroxy-3-naphthoic acid (I) was obtained using the same procedure, except that formation of the allyl amide was achieved in better yields through the acid chloride. When 1-hydroxy-2-naphthoic acid was subjected to these reactions, the yields were much lower.



(I)



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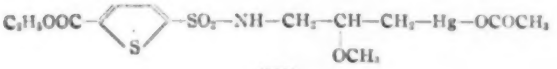


(III)

The preparation of a thiophene analog of mersalyl involved first the formation of the allyl amide of 2-thiophenesulfonic acid. It was hoped that the mercurated product (II) would be alkali-soluble because of the remaining hydrogen atom on the amide nitrogen. This, however, was not the case. This synthesis was also complicated by the fact that α-mercuration of the ring took place at elevated temperatures. To avoid these difficulties the allyl sulfonamide of 2-thenoic acid was prepared and mercurated (III). To avoid destruction of the carboxyl group on mercuration, according to the equation:

$$R{-}COOH \rightarrow R{-}COOHg{-}OOC{-}CH_3 \rightarrow R{-}HgOOC{-}CH_3 + CO_2$$

the ethyl ester was employed, and the mercurated ester sulfonamide was found soluble in dilute sodium hydroxide. On treating with excess dilute alkali, or heating, however, degradation took place with loss of mercury from the molecule. Attempts to hydrolyze the ester without affecting the mercury linkage were unsuccessful.

An acidic solubilizing group on a thiophene analog of mersalyl was introduced, however, by first preparing the half-acid ester of 2,5-dicarboxythiophene on treatment of the diester with the required amount of alcoholic potassium hydroxide. This was possible because the potassium salt of the half ester is insoluble in ethanol. The formation of the allyl amide and mercurated derivative was readily achieved to give an amido ester which could be hydrolyzed to the free acid (IV) by gentle treatment with aqueous potassium hydroxide without removal of the mercury. The product, when freshly prepared, was soluble in dilute sodium bicarbonate solution.

The naphthalene analog was given intravenously to dogs in a dose of 0.75 mg. Hg per Kg. of body weight, and the diuretic effect[1] expressed as increased urine output over normal for a three-hour period in cc./Kg. of body weight was 2.5, which compares favorably with commercial diuretics. It was necessary to introduce the thiophene analogs directly into the small intestine of the dogs in order to by-pass the stomach, but indications of diuresis were seen in each instance. The acute oral toxicities in rats, expressed as median lethal doses ± standard errors, are presented for these compounds (Table I), but have limited usefulness because oral administration of mercurials is generally unsatisfactory.

EXPERIMENTAL[2]

N-Allylsalicylamide.—Salicyclic acid (55 Gm.) and allyl isothiocyanate (50 Gm.) were refluxed for twenty hours, the mixture was vacuum distilled, and the distillate from 130–140° was collected. The yellow oil was purified by dissolving in ether and extracting with dilute bicarbonate and then dilute sodium hydroxide. The latter extract was neutralized, and the yellow oil was taken up in ether, dried, and distilled. The yield was 17.6 Gm. (25%).

***o*-(N-Allylcarbamyl)-phenoxyacetic Acid.**—The amide (8 Gm.) from the previous step was reacted with chloroacetic acid (4.5 Gm.) in the presence of sodium hydroxide (5 Gm. in 60 ml. of water). The mixture was refluxed for one hour and then neutralized. The product appeared as an oil, but became crystalline on prolonged scratching. It was

[1] We are indebted to Drs. K. K. Chen and E. B. Robbins of the Lilly Research Laboratories for the reports of the diuretic activity of these compounds.

[2] The carbon-hydrogen analyses were carried out by the Clark Microanalytical Laboratory, Urbana, Ill. The mercury analysis was that described for assay of mersalyl in the U. S. Pharmacopeia, Fourteenth Revision.

recrystallized from dilute methanol to give 4.3 Gm. (40%) of product melting at 118–119°, which agrees with the reported value (5).

o - [N - (γ - Acetoxymercuri - β - methoxypropyl)-carbamyl]-phenoxyacetic Acid.—The o-(N-allylcarbamyl)-phenoxyacetic acid (3 Gm.) in methanol (30 ml.) was mixed with mercuric acetate (4 Gm.) in methanol (60 ml.) and allowed to stand overnight. The product separated as an oil, and after the methanol was decanted, the oil was ground under water until it became crystalline. The yield was 3.3 Gm. (50%).

Anal.—Calcd. for $C_{15}H_{19}O_7NHg$: Hg, 41.4. Found: Hg, 43.0.

2-Hydroxy-3-N-allylnaphthoylamide.—The sodium salt of 2-hydroxy-3-naphthoic acid (10 Gm.) was suspended in dry benzene (50 ml.) and treated slowly with thionyl chloride (10 ml.). The reaction was stirred for an hour at room temperature, the sodium chloride was filtered and washed with benzene, and the excess thionyl chloride and benzene were removed under reduced pressure. The acid chloride was obtained as a yellow crystalline mass in a yield of 85% (8.4 Gm.), m.p. 95–96°.

The acid chloride (8.4 Gm.) was dissolved in dry benzene (50 ml.) and treated with an excess of allyl amine (3 Gm.) dropwise with constant swirling. After allowing the reaction to cool for an hour, the solution was washed with water and extracted with 10% sodium hydroxide. On neutralization, 8.3 Gm. (88%) of the amide was obtained, m. p. 118–119°.

2-Oxyacetic Acid-3-N-allylnaphthoylamide.—The 2-hydroxy-3-N-allylnaphthoylamide (6 Gm.) was dissolved in 40 ml. of water containing sodium hydroxide (2.2 Gm.) and treated with chloroacetic acid (2.5 Gm.) in 20 ml. of water. After heating on a water bath for an hour, the reaction was neutralized, and the precipitate was taken up in ether. The ether solution was extracted with dilute alkali and the extract was neutralized to give a colorless product, which, after recrystallization from absolute methanol, weighed 1.1 Gm. (15%), and melted at 206–207°.

Anal.—Calcd. for $C_{16}H_{15}NO_4$: C, 67.47; H, 5.26. Found: C, 66.81; H, 5.45.

2-Oxyacetic Acid-3- [N - (γ - acetoxymercuri - β - ethoxypropyl)]-naphthoylamide (I).—A solution of the previous amide (5.1 Gm.) in absolute ethanol (25 ml.) was heated to boiling, and a hot solution of mercuric acetate (5.7 Gm.) in ethanol was added. After remaining at room temperature overnight, most of the solvent was removed under reduced pressure, and the resulting oil was scratched under distilled water until crystallized. The yield was 5.5 Gm. (40 %).

Anal.—Calcd. for $C_{20}H_{23}NO_7Hg$: C, 40.71; H, 3.93. Found: C, 39.81; H, 3.59.

2-Thiophenesulfonyl Chloride.—Chlorosulfonic acid (100 Gm.) was cooled by an ice-salt bath, and thiophene (25 Gm.) in 50 ml. of chloroform was added gradually with stirring. After two additional minutes, the reaction was poured into ice, and 50 ml. more of chloroform was added. After vigorous stirring, the chloroform layer was washed well with water and saturated salt solution, and dried over calcium chloride. The solution was distilled under reduced pressure, the sulfonyl chloride distilling at 125–130° at 15 mm. It crystallized on cooling to give 22 Gm. (44%) of glistening needles, m. p. 32–33°, which agrees with the reported value (6).

2 - N - Allylthiophenesulfonamide.—2 - Thiophenesulfonyl chloride (22 Gm.) dissolved in 30 ml. of dry benzene was treated drop by drop with allyl amine (10 Gm.) in 20 ml. of benzene with frequent swirling. After allowing the reaction to stand for three hours, the benzene solution was washed with 10% hydrochloric acid and distilled water. The sulfonamide was then extracted with 10% sodium hydroxide, and on neutralization, white crystals were collected, washed with water, and dried. The yield was 21 Gm. (85%), m. p. 54–56°.

Anal.—Calcd. for $C_7H_9NO_2S_2$: C, 41.35; H, 4.46. Found: C, 41.53; H, 4.43.

2 - [N - (γ - Acetoxymercuri - β - methoxypropyl)]-thiophenesulfonamide (II).—The thiophenesulfonamide (5 Gm.) was dissolved in 30 ml. of dry methanol, and a hot solution of 7.8 Gm. of mercuric acetate in 70 ml. of methanol was added gradually. The mixture was heated for several hours on a steam bath and then allowed to stand overnight. After evaporation of the solvent, a viscous mass was obtained which crystallized after prolonged scratching under distilled water. A yield of 6 Gm. (50%) was obtained.

Anal.—Calcd. for $C_{10}H_{13}NO_5S_2Hg$: C, 24.31; H, 3.06. Found: C, 22.92; H, 2.65.

2-Thenoic Acid and Thiophene-2,5-dicarboxylic Acid.—The procedure of Campaigne and Foye (7) was used to prepare these acids both of which were obtained in yields approximating 80%.

Ethyl 2-Thenoate and Diethyl Thiophene-2,5-dicarboxylate.—The esters were readily prepared in yields of 85–90% by the Fischer-Speier method. The ethyl 2-thenoate boiled at 115–116° at 16 mm., and the dicarboxy ester melted at 50–51°.

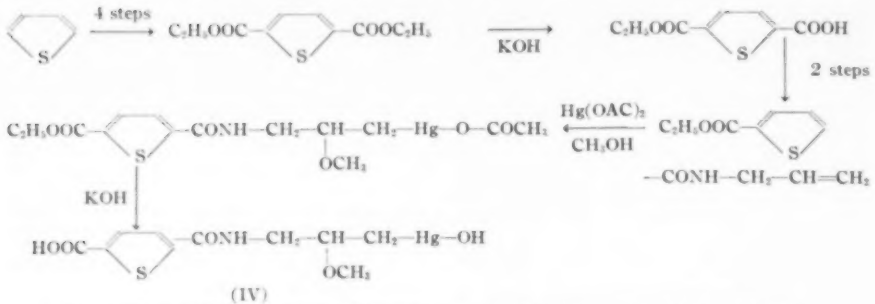
TABLE I.—ACUTE ORAL TOXICITIES OF THE MERSALYL ANALOGS

Compound	LD_{50} ± S. E., Mg./Kg.
I	598 ± 156
II	128 ± 19
III	741 ± 93
IV	968 ± 180

2-Carbethoxy-5-thiophenesulfonyl Chloride.—Ethyl 2-thenoate (10 Gm.) in 50 ml. of chloroform was reacted gradually with an excess of chlorosulfonic acid (50 Gm.). After refluxing gently on a steam bath for one-half hour, the reaction mixture was poured into ice, and the chloroform layer was washed with water and dried. The product was distilled under reduced pressure, b. p. 180–185° at 15 mm., and 6.5 Gm. (40%) of the oil was obtained.

2 - Carbethoxy - 5 - N - allylthiophenesulfonamide.—To the sulfonyl chloride (6.5 Gm.) in 25 ml. of dry benzene was added gradually an excess of allyl amine (2 Gm.) with cooling between additions. After cooling for an hour, the benzene solution was washed with water and then extracted with 10% sodium hydroxide. White crystals were obtained on neutralization and were washed repeatedly with cold water. The yield was 5 Gm. (71%); m. p. 150–152°.

Anal.—Calcd. for $C_{10}H_{13}NO_4S_2$: C, 43.62; H, 4.76. Found: C, 42.82; H, 4.04.



Eq. 1.—Synthesis of 2-Carboxy-5-[N-(γ-hydroxymercuri-β-methoxypropyl)]-thenoylamide.

2 - Carbethoxy - 5 - [N - (γ - acetoxymercuri - β-methoxypropyl)]-thiophenesulfonamide (III).—The ester sulfonamide (5 Gm.) obtained in the previous step was boiled in methanol (30 ml.) and treated with a hot solution of mercuric acetate (5.6 Gm.) in methanol (60 ml.), whereupon immediate precipitation took place. After standing for twenty hours at room temperature, the mixture was diluted with 50 ml. of water and filtered. The yield of white solid was 7.6 Gm. (75%); m. p. 166–167°.

Anal.—Calcd. for $C_{13}H_{19}NO_7S_2Hg$: C, 27.58; H, 3.38. Found: C, 27.41; H, 3.52.

2-Carbethoxy-5-thenoic Acid.—Diethyl thiophene-2,5-dicarboxylate (9.5 Gm.) was dissolved in 50 ml. of absolute ethanol, and to the vigorously stirred solution was gradually added 2.4 Gm. of potassium hydroxide in 50 ml. of ethanol. The potassium salt of the half ester precipitated in a few minutes. The stirring was continued for two hours, and the product was filtered, dissolved in water, and precipitated by dilute hydrochloric acid. The half-acid ester was taken up in ether, extracted with dilute bicarbonate, and reprecipitated with dilute acid. After washing with water and drying, 7 Gm. (83%) of colorless solid was obtained, m. p. 140–142°.

2-Carbethoxy-5-thenoyl Chloride.—The half-acid ester (9 Gm.) was suspended in an excess of thionyl chloride (25 ml.), and the solution was refluxed gently for one-half hour. The product was distilled under reduced pressure, boiling at 132–135° at 10 mm., and melting at 38–40°. The yield was 8 Gm. (80%).

2-Carbethoxy-5-N-allylthenoylamide.—The allyl amide was easily prepared in the usual manner using allyl amine in benzene. The product, after recrystallization from benzene, was obtained in 92% yield and melted at 117–118°.

Anal.—Calcd. for $C_{11}H_{13}NO_3S$: C, 55.21; H, 5.47. Found: C, 55.24; H, 5.65.

2 - Carboxy - 5 - [N - (γ-hydroxymercuri - β-methoxypropyl)]-thenoylamide (IV).—The mercuration took place in the usual manner to give an oil which was crystallized by scratching under cold water. The white crystals were obtained in 76% yield and melted at 99–101°.

The mercurated ester amide (6.5 Gm.) was suspended in 50 ml. of water, and potassium hydroxide (2.5 Gm.) was added gradually with stirring. The compound formed a solid plastic mass, but dissolved on gradual heating. The solution was cooled, filtered, and neutralized with dilute acetic acid to give a white precipitate. The yield was 3 Gm. (50%) of solid, soluble in dilute sodium bicarbonate, which had no sharp melting point, but softened at 143°.

Anal.—Calcd. for $C_{10}H_{11}NO_5SHg$: C, 26.11; H, 2.84. Found: C, 25.94; H, 2.35.

SUMMARY

1. Detailed directions for the synthesis of mersalyl and several naphthalene and thiophene analogs are presented. Use has been made of both carbonamide and sulfonamide linkages to join the mercurated portion of the molecule with aromatic nuclei.

2. The following new compounds have been prepared: 2-hydroxy-3-N-allylnaphthoylamide, 2-oxyacetic acid-3-N-allylnaphthoylamide, 2-oxyacetic acid-3-[N-(γ-acetoxymercuri-β-ethoxypropyl)]-naphthoylamide, 2-N-allylthiophenesulfonamide, 2-[N-(γ-acetoxymercuri-β-methoxypropyl)]-thiophenesulfonamide, 2-carbethoxy-5-N-allylthiophenesulfonamide, 2-carbethoxy-5-[N-(γ-acetoxymercuri-β-methoxypropyl)] - thiophenesulfonamide, 2-carbethoxy-5-thenoic acid, 2-carbethoxy-5-N-allylthenoylamide, 2-carboxy-5-[N - (γ-hydroxymercuri-β-methoxypropyl)]-thenoylamide.

3. The mercurated compounds were found to possess diuretic activity but only the naphthalene diuretic was sufficiently soluble in alkali to be administered intravenously.

REFERENCES

(1) Brunn, F., *Wien. klin. Wochschr.*, **37**, 901(1924).
(2) Tabern, D., U. S. pat. 2,300,992 (1942).
(3) Hartmann, M., U. S. pat. 2,136,501 (1938).
(4) Robbins, E. B., and Chen, K. K., THIS JOURNAL, **40**, 249(1951).
(5) Bockmühl, M., and Schwarz, A., German pat. 423,031 (1924).
(6) Steinkopf, W., and Hopner, T., *Ann.*, **501**, 174(1933).
(7) Campaigne, E. E., and Foye, W. O., *J. Am. Chem. Soc.*, **70**, 3941(1948).

The Determination of Histamine in Pharmaceutical Products by Means of 2,4-Dinitrofluorobenzene*

By FLOYD C. McINTIRE

A simple, precise, and highly reproducible method is presented for the determination of histamine in pharmaceutical histamine preparations. This method is based upon the formation of the very stable N-α-(2,4-dinitrophenyl) histamine from the reaction between histamine and 2,4-dinitrofluorobenzene.

CHEMICAL METHODS presently employed by control laboratories for the determination of histamine in pharmaceutical histamine preparations leave much to be desired with respect to precision, reproducibility, and stability of the colored histamine derivatives. The method presented here has been found to have a high degree of precision and reproducibility. The histamine derivative upon which this method is based is very stable. The procedure which follows is a simplification of a method for the determination of histamine in biological materials (1); the chemistry and specificity of the method were discussed in the earlier publication.

REAGENTS

(*a*) 2,4-Dinitrofluorobenzene (DNFB) [prepared by the method of Cook and Saunders (2)] dissolved in absolute alcohol—0.15 ml. of DNFB in 25 ml. of alcohol. A fresh solution should be made each week. DNFB melts at 30° and is most conveniently measured volumetrically in the liquid state.

(*b*) Low-copper water—distilled water which has a very low content of Cu^{++} or Cu^{+}.

(*c*) Sodium diethyldithiocarbamate (Eastman) (NaDi), 0.04 *M*, 6.84 mg./ml., in low-copper water.

(*d*) Carbonate buffer, *p*H ~10.2, in low-copper water, prepared by mixing equal volumes of *M* sodium carbonate and *M* sodium bicarbonate.

(*e*) Hydrochloric acid, 0.11 *N* in water.

(*f*) Benzene, analytical reagent grade.

PROCEDURE

Dilute the sample to be analyzed for histamine with low-copper water, carbonate buffer, and NaDi solution to give a final solution which contains 20–100 μg. of histamine base per ml. and which is 0.1 *M* to the carbonate ion and 0.004 *M* to NaDi (buffered histamine solution). To 1 ml. of the buffered histamine solution add 1 ml. of DNFB solution and mix well. After twenty minutes at room temperature, dilute the histamine-DNFB mixture to 20 ml. with the 0.11 *N* aqueous hydrochloric acid. In a separatory funnel extract the acidified solution with an equal volume of benzene. Measure the optical density of the aqueous phase at 358 mμ.

With each set of determinations, a reaction blank and at least two different concentrations of histamine standard should be run. Figure 1 shows the relationship between the optical density reading and histamine concentration in the final solution. These are data from a typical experiment and indicate the high degree of reproducibility of the method.

There is a possibility of interference by sufficiently high concentrations of phenol. In the assay of extremely dilute histamine preparations which contain phenol as a preservative, it may be necessary to remove most of the phenol by extracting the sample with benzene or ether before proceeding with the assay.

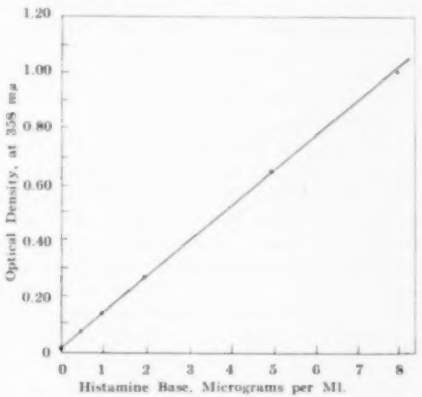


Fig. 1.—Relationship between histamine concentration and optical density reading.

CALCULATION

The final solution represents a dilution of 1 → 20 from the buffered histamine solution. Therefore:

$$\left(\begin{matrix}\text{Original concentration of histamine base in sample}\end{matrix}\right) = \left(\begin{matrix}\text{Final concentration calculated from optical density reading}\end{matrix}\right) \times 20 \times \left(\begin{matrix}\text{Dilution factor in buffered histamine solution}\end{matrix}\right)$$

For example, a U. S. P. histamine diphosphate solution, which was diluted 1 → 25 in the buffered histamine solution and which gave an optical density reading corresponding to 2 μg. of histamine base per ml., would contain 2 × 25 × 20 μg., or 1 mg., of histamine base per ml. of the original solution.

REFERENCES

(1) McIntire, F. C., White, F. B., and Sproull, M., *Arch. Biochem.*, **29**, 376(1950).

(2) Cook, H. G., and Saunders, B. C., *Biochem. J.*, **41**, 558 (1947).

* Received November 8, 1951, from the Research Division, Abbott Laboratories, North Chicago, Ill.

A Colorimetric Assay for Pilocarpine and Its Salts*

By JOHN W. WEBB,† RAY S. KELLEY,‡ and ARTHUR J. McBAY§

Shupe's colorimetric method for the determination of pilocarpine has been found applicable, with modifications, to the quantitative determination of pilocarpine hydrochloride and pilocarpine nitrate. A comparison of the results obtained on commercially available samples by this method with those obtained by both a micro-Kjeldahl determination and the A. O. A. C. method indicates the suitability of this assay for the quantitative determination of pilocarpine and its salts.

A qualitative test for pilocarpine was first developed by Helch (1) in 1902. This test is based on the property of pilocarpine to form a violet-colored compound in the presence of slightly acid hydrogen peroxide and potassium dichromate. This violet-colored compound is soluble in chloroform and in benzene. It is not to be confused with the blue perchromic acids formed by peroxide and dichromate in strong acid media. The latter are insoluble in benzene and in chloroform. The Helch test, with benzene as the solvent, is official in the United States Pharmacopeia as an identity test for pilocarpine and its salts.

Most of the quantitative assays for pilocarpine and its salts have serious disadvantages. Any volumetric assay of pilocarpine in an aqueous medium will offer difficulty, since pilocarpine and its salts when present in the same solution constitute a buffer system. The A. O. A. C. assay is of this type with methyl red as the indicator. The volumetric method has other disadvantages: the alkaloid is freely soluble in water, thus making its extraction with chloroform difficult; the alkaloid deteriorates in excess amounts of alkali; it is subject to decomposition by oxidation; prolonged heating at a temperature above 100° or the presence of moisture may cause decomposition; and the assay requires a considerable amount of time.

An assay for nitrogen by the Kjeldahl method may or may not serve as an accurate assay for pilocarpine, for the pilocarpine could be partly decomposed and still have the proper nitrogen content.

The Helch test (1) has been modified by Shupe (2) in his development of a quantitative colorimetric assay. In his modification, pilocarpine is extracted from an ammoniacal solution of one of its salts and is then treated to develop the color.

Since pilocarpine is easily decomposed, it was believed that an assay of pilocarpine salts could be developed in which the liberation of the unstable free base could be avoided.

Higuchi and Concha (3) titrate pilocarpine hydrochloride and pilocarpine nitrate in glacial acetic acid with perchloric acid, using methyl violet and α-naphtholbenzein, respectively, as indicators.

EXPERIMENTAL

In 1903, Jowett (4) reported the absorption spectra of pilocarpine and pilocarpine nitrate; on the basis of this work, direct readings of the ultraviolet absorption spectra of pilocarpine and pilocarpine salts were attempted.

Both pilocarpine and pilocarpine nitrate showed a maximum in their respective absorption curves, but with the concentrations of pilocarpine hydrochloride used, no maximum could be found. Since ultraviolet absorption curves of the alkaloid and the two commonly used salts seemed to offer no possibility of correlation, this method of assay was discontinued in favor of one in which a color is developed. With certain modifications the assay selected was that employed by Shupe (2).

Shupe has stated that the violet color which was developed by his method of assay has its greatest density at a wave length of 560 mμ. Figure 1 shows the absorbance wave-length curve of the violet color. Inspection of the curve indicates that the optimum wave length is 560 mμ.

PROCEDURE

The alkaloid and two of its salts were investigated. Each was dissolved in water so as to make a concentration of about 2 mg./cc. Aliquots representing 2, 4, and 6 mg. of the sample were placed in 125-cc. separatory funnels, and water was added to make a total volume of 5 cc. To each sample was added 1 cc. of 20% acetic acid, 10 cc. of chloroform, 1 cc. of 5% potassium chromate solution, and 2 cc. of 3% hydrogen peroxide. The mixtures were immediately extracted with chloroform. Extraction was performed by shaking the mixture for ninety seconds. The chloroform phase was filtered through cotton into a 25-cc. glass-stoppered volumetric flask. The process was repeated with each sample with a 10-cc. and then with a 5-cc. portion of chloroform.

* Received August 31, 1951, from the Samuel M. Best Research Laboratory, Massachusetts College of Pharmacy, Boston.

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Abstracted from a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by John W. Webb, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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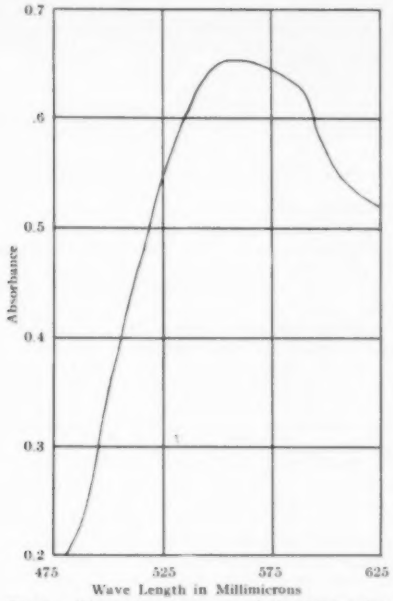

Fig. 1.—Absorption spectrum of the violet-colored pilocarpine perchromate (4.9 mg. of pilocarpine/cc.). Beckman spectrophotometer, 1-cm. cell, slit width 0.2 mm.

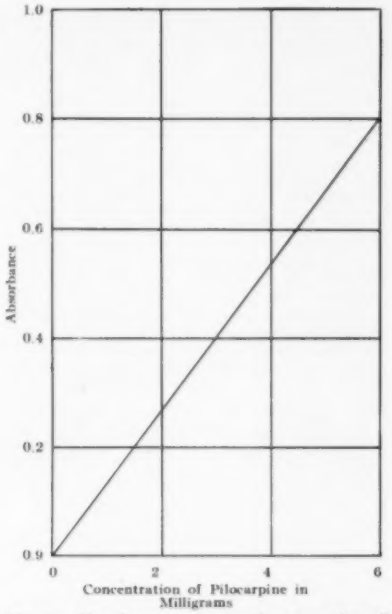

Fig. 2.—Absorbance-concentration curve for pilocarpine. Beckman spectrophotometer, 1-cm. cell, wave length 560 mμ, slit width 0.2 mm.

The total chloroform extracts were brought to the 25-cc. mark in the volumetric flask and mixed well, and the intensity of the violet color was measured in a 1-cm. cell by means of a Beckman spectrophotometer set at a slit width of 0.2 mm. and at a wave length of 560 mμ.

As stated by Shupe (2), the color is sensitive to light; therefore the best results are obtained when readings are made as soon as possible after the color development so as to avoid errors from temperature changes and evaporation.

TABLE I.—ASSAY RESULTS

	A. O. A. C. Method, Pilocarpine, %	Colorimetric Method, Pilocarpine, %
Pilocarpine	56.50	56.34
Pilocarpine HCl I	80.80	80.17
Pilocarpine HCl II	84.81	83.00[a]
Pilocarpine HNO_3	76.17	76.17[b]

[a] Kjeldahl method gave 83.66%.
[b] A. U. S. P. sample of pilocarpine nitrate assayed by the A. O. A. C. method was used as a standard for the colorimetric method.

The violet color of the compound, characterized by Biedebach (5) as pilocarpine perchromate, fades when the *p*H is lowered and is eventually replaced by the blue of the perchromic acids. The optimum *p*H, 2.9, has been established, and may be obtained by using the specified concentration of acetic acid. It has also been established that at the optimum *p*H the absorbance-concentration curve, Fig. 2, adheres to Beer's law.

The results of the assay of the three most common forms of pilocarpine are presented in Table I.

SUMMARY AND CONCLUSIONS

1. A method has been described for a direct quantitative colorimetric assay of pilocarpine, pilocarpine nitrate, and pilocarpine hydrochloride.

2. The colorimetric method has the following advantages:

(*a*) The difficulty in the A. O. A. C. method of determining the end point is eliminated.

(*b*) This method requires less time than either a Kjeldahl or the A. O. A. C. method.

(*c*) The formation of the unstable alkaloid is avoided.

(*d*) The reagents need be only of approximate strength.

(*e*) Determinations of pilocarpine present in weak concentrations may be made.

REFERENCES

(1) Helch, M. H., *Pharm. Post*, **35**, 289(1902).
(2) Shupe, I. S., *J. Assoc. Offic. Agr. Chemists*, **24**, 757 (1941).
(3) Higuchi, T., and Concha, J., THIS JOURNAL, **40**, 173 (1951).
(4) Jowett, H. A. D., *J. Chem. Soc.*, **83**, 453(1903).
(5) Biedebach, F., *Apoth. Ztg.*, **49**, 723(1933).

The Determination of Carvone in Oil of Spearmint*

By LaVERNE D. SMALL, JAMES E. DUSENBERRY, and WALTER T. GLOOR, Jr.

The time required by the official method for determining the carvone content of spearmint oil can be considerably shortened by using a modified apparatus incorporating a magnetic stirrer. A spectrophotometric method is also presented, using solutions of different concentrations of carvone in "carvone-free" spearmint oil and diluting with 95 per cent ethyl alcohol; a graph can thus be prepared by plotting concentration against absorbancy at the wave length of maximum absorption.

A NEUTRAL SULFITE METHOD of assay is described in the U. S. P. XIV (1) for the determination of the carvone content of spearmint oil. This is a time-consuming assay when using the equipment described in the U. S. P. XIV. In addition to the long time required, there is a possibility of loss of some of the volatile constituents of the oil during the assay. Such a loss of material would, by the assay procedure described, be calculated as carvone. This would result in a higher apparent carvone content.

This paper serves two purposes: first, to describe a modified apparatus that shortens the time required to complete the assay by as much as 50 per cent, minimizes the possibility of loss of any volatile constituents, and simplifies the operation of the assay; and second, to show the results of a preliminary study of the carvone content of spearmint oil by means of an ultraviolet spectrophotometer.

Cooke and Macbeth (2) reported that carvone, when dissolved in purified ethanol, has two absorption maxima; the one of greater intensity occurs at 235.3 mμ, while a much weaker maximum occurs at 317.5 mμ.

A pure sample of carvone was prepared by the method described by Cooke and Macbeth (2). This purified carvone was used to determine the molecular extinction coefficient in alcohol at 235 mμ. It was also mixed with definite amounts of specially prepared carvone-free spearmint oil to approximate, as closely as possible, natural spearmint oil containing a known amount of carvone. The absorbancies of carvone in these standardized spearmint oil solutions were determined and used to prepare the standard curve for carvone in spearmint oil shown in Fig. 2.

* Received August 31, 1951, from the College of Pharmacy, University of Nebraska, Lincoln.
Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.

THE NEUTRAL SULFITE METHOD

Apparatus

Figure 1 shows the complete modified apparatus for the determination of carvone by the neutral sulfite method. A standard 100-ml. Pyrex volumetric cassia flask was modified by rounding the bottom of the flask. This permitted using a regular 100-ml. Glas-Col heating mantle. The standard taper ground neck was replaced by one of larger size (14/20), into which was fitted a 10-cm. water-jacketed condenser. Just above the water jacket on the condenser, a small curved air vent may be seen. Into the top of the condenser, fitted with a standard taper 12/30 ground joint, a small dropping funnel was placed with the tip extending into the condenser below the air vent. The dropping funnel was made from a 50-ml. distilling flask to which a standard Pyrex stopcock was attached. The reaction mixture was stirred continually during the heating period by means of a magnetic stirrer. The temperature of the heating mantle was shown by a pyrometer and regulated by a Powerstat variable transformer. The total height of the apparatus, as pictured, was 68 cm.

Procedure

The neutral sulfite method as given by the U. S. P. XIV (1) was followed. The results of analyses of four samples of spearmint oil, using the apparatus described in the U. S. P. XIV and the modified apparatus shown in Fig. 1, are shown in Table I. Oil samples 3 and 4 were both very old and had not been stored properly.

SPECTROPHOTOMETRIC STUDY

A Beckman Model DU spectrophotometer was used for the spectrophotometric study. A pair of 1.000-cm. matched silica cells was used. The slit opening was set at 0.75 mm. and the temperature of the room in which the measurements were made was controlled at 24–25°. All volumetric measurements were made at 20°.

Materials

Solvent.—The ethyl alcohol used as a solvent was purified by a method given by Leighton, Crary, and Schipp (3) wherein the ethyl alcohol U. S. P. was refluxed with 12 *N* sulfuric acid and distilled, and then was refluxed with potassium hydroxide and silver nitrate, and again distilled.

Carvone.—A commercial sample of carvone[1] was purified by the method used by Cooke and Macbeth (2), in which a carefully fractionated sample of carvone was converted into the hydrosulfide derivative. The ketone was regenerated from this deriva-

[1] The carvone was obtained from Fritzsche Bros., Inc., New York, N. Y.

TABLE I.—CARVONE PERCENTAGE IN SAMPLES OF SPEARMINT OIL, OBTAINED WITH U. S. P. XIV AND MODIFIED APPARATUS

Sample No.	1	2	3	4
U. S. P.	55.0	69.0	51.0	54.0
	54.0	69.0	52.0	53.0
Modified	55.0	68.5	50.0	53.0
	54.0	68.5	49.5	52.0

tive by treatment with alcoholic potassium hydroxide followed by steam distillation. The carvone had the following properties: n^{25}_{D} 1.4985; d^{25}_{4} 0.9626. These values compare favorably with those given by Guenther (4).

A commercial sample of carvone was used because of its availability. Although this carvone was obtained from caraway oil and was the *d*-isomer, it was adequate for the purposes of this study, since optical isomerism does not influence the ultraviolet absorption.

Carvone-Free Spearmint Oil.—The neutral sulfite method given by Guenther (5) was used to remove the carvone from a sample of spearmint oil. The procedure and equipment were adapted to a 150-ml. sample. The unreacted oil from this reaction was separated from the aqueous solution, washed with water, and dried over Drierite. The oil was filtered, and was stored in a Pyrex glass-stoppered flask. The density of this oil was 0.8781 (d^{25}_{4}). A sample containing 28.1 γ/ml. in ethyl alcohol when examined in the spectrophotometer showed no absorption peak, but only a gradual increase in transmittance from 228 mμ to 255 mμ.

Procedure

Determination of Carvone in Standardized Samples of Spearmint Oil.—The samples were prepared by weighing (to the nearest 0.1 mg.) definite amounts of carvone and mixing with exact amounts of the carvone-free spearmint oil. The percentage (v/v) of carvone in these solutions was calculated by using the densities of the carvone and the carvone-free oil. These solutions were diluted by measuring 1.0 ml. of the standard sample with a Normax-grade delivery pipette and diluting to 250.0 ml. with purified ethyl alcohol. One milliliter of this primary dilution was then diluted to 250.0 ml. This results in a 1:62,500 dilution of the standard sample and brings the carvone to a concentration that may be readily measured by the spectrophotometer. The absorbancies of these solutions were determined at 235 mμ and are shown in Table II. These absorbancies plotted against percentage (v/v) gave the standard curve shown in Fig. 2.

The percentage of carvone in the spearmint oils used in the neutral sulfite method was determined

TABLE II.—VALUES DETERMINED FOR CARVONE ADDED TO CARVONE-FREE SPEARMINT OIL FROM TRANSMITTANCY AT 235 mμ

% Carvone, v/v	As	Log ϵ
47.62	0.4622	3.976
53.53	0.5114	3.969
59.93	0.5638	3.962
65.03	0.5969	3.952
66.34	0.6108	3.953

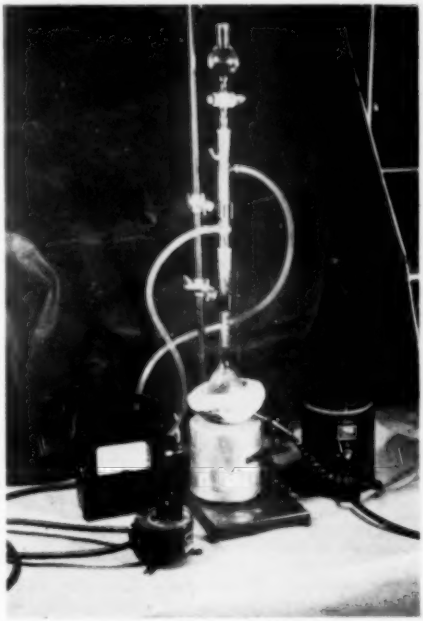

Fig. 1.—Neutral sulfite method. Showing the modified apparatus.

by means of the spectrophotometer. The spearmint oils were diluted, as were the standard samples. The absorbancies of these solutions were determined. When applied to the standard curve in Fig. 2, the following carvone concentrations were obtained: sample No. 1, 52.8%; No. 2, 68.0%; No. 3, 47.6%; No. 4, 47.7%.

An alcoholic solution of carvone-free spearmint oil was prepared, representing the concentration of noncarvone constituents in a sample of spearmint oil containing 60% carvone. It showed 96.7% transmittancy, or an absorbancy of 0.0146.

Cooke and Macbeth (2) reported that pure carvone in alcohol at 235 mμ has a log ϵ 4.28 (log of the molecular extinction coefficient). A sample of pure carvone, prepared for this study, was diluted with purified ethyl alcohol to make four different concentrations ranging from 7.9 γ/ml. to 19.8 γ/ml. The absorbancies of these solutions were determined and the following values for log ϵ were obtained: 3.953, 3.953, 3.954, and 3.952.

DISCUSSION

The results obtained by using the modified apparatus for the neutral sulfite method are comparable with the results obtained with the equipment described in U. S. P. XIV (1). In most samples, the percentages of carvone obtained were a little lower using the modified apparatus. This may be due to the loss of some of the more volatile constituents of the oil when using the standard apparatus.

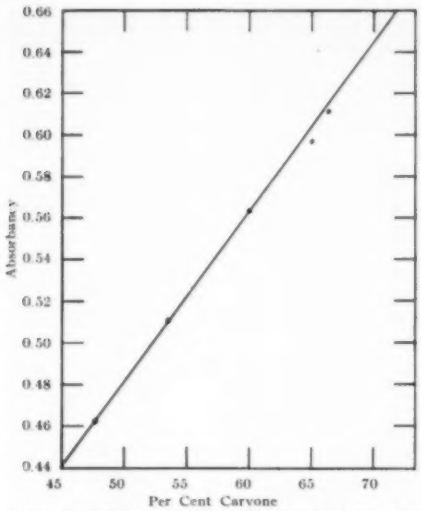

Fig. 2.—Spectrophotometric method. A standard curve showing the relationship of percentage (v/v) of carvone to absorbancy at 235 mμ.

The spectrophotometric study shows that an assay for carvone in spearmint oil could probably be developed. A standard curve similar to the one shown in Fig. 2 would probably be most practicable. Such a curve would take into account the varying effect of the noncarvone portion of the oil. This effect upon the apparent absorbancy of the carvone would be greater in those oils having a lower carvone content. This would explain the higher values for log ϵ from the oils with lower percentages of carvone.

By calculating the percentage of carvone in the spearmint oil sample No. 1 from the absorbancy obtained in this study, using the value log ϵ 4.28, we find 25.8% as compared with 52.8% using the standard curve in Fig. 2. If instead of log ϵ 4.28 the value log ϵ 3.954 is substituted, the concentration of carvone in this oil would be 54.7%. From the data thus far obtained, it would appear that log ϵ for carvone in alcohol at 235 mμ is much closer to 3.95 than it is to 4.28.

Many more samples of spearmint oil and of carvone in alcohol must be checked before a satisfactory assay procedure using the ultraviolet spectrophotometer is developed and before log ϵ for carvone is definitely established.

SUMMARY

An improved apparatus for the determination of carvone in spearmint oil by the neutral sulfite method has been developed. By using this equipment, the time required to complete the assay is greatly shortened, the loss of volatile constituents of the oil during the assay is prevented, and the operation of the assay is simplified.

A spectrophotometric method of determining the carvone content of spearmint oil has been studied. A standard curve was prepared showing percentage carvone concentration versus absorbancy at the wave length maximum absorption, 235 mμ. The percentages of carvone in four samples of spearmint oil when determined from this curve were lower than those determined by the U. S. P. XIV method.

A preliminary investigation showed that log ϵ 3.954 for carvone in alcohol at 235 mμ, when used to calculate the percentage of carvone in spearmint oil, gave a value that was much closer to the actual percentage than if the reported value of log ϵ 4.28 was used.

Investigation of this subject is being continued.

REFERENCES

(1) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950, p. 565.
(2) Cooke, R., and Macbeth, A., *J. Chem. Soc.*, **1938**, p. 1408.
(3) Leighton, P., Crary, R., and Schipp, L., *J. Am. Chem. Soc.*, **53**, 3017(1931).
(4) Guenther, E., "The Essential Oils," Vol. 2, D. Van Nostrand Co., New York, 1949, p. 414.
(5) Guenther, E., "The Essential Oils," Vol. 1, D. Van Nostrand Co., New York, 1948, p. 238.

Note

A Note on an Apparatus for Prolonged and Repeated Intravenous Infusions in Large Animals*, †

By LAWRENCE E. DETRICK and BONNIE RHODES‡

PROLONGED and repeated infusions into the external jugular vein, with the usual indwelling polyethylene catheter apparatus, was accompanied by considerable difficulty. A fine white precipitate formed within the capillary tubing which occluded the flow of the nutrient solution administered at a rate of 25 cc. per hour with 125 cm. hydrostatic pressure. A leather collar for external protection was not satisfactory. Both of these difficulties were overcome by a slight modification of the indwelling venous catheter apparatus reported by Aurin and Herrmann (1).

Figure 1 illustrates the modification made in previously reported catheters that overcame the complications encountered. Polyethylene tubing[1] (*A*) has an i. d. of 0.03 in. and an o. d. of 0.049 in., fits tightly over the shaft of hypodermic needle (*C*), and is from 5 1/4 in. to 5 1/2 in. long. Tubing (*B*) has an i. d. of 0.045 in., an o. d. of 0.062 in., a length of 0.75 in. and fits tightly over both the metal needle shaft and tubing (*A*). The hypodermic needle (*C*) is a Becton-Dickinson 19-gauge needle with a metal shaft length of 0.5 in. and is threaded at the syringe adapter end (threads = 1/4 in. diameter x 28 tip). A corner of the needle has been perforated to anchor the assembly to the neck skin with steel wire. Stylet (*D*) is made of nylon "fishing leader" (o. d. = 0.021 in.), with one end embedded in a rubber stopper (stopper for a 1/4-cc. vial) which tightly closed the syringe adapter end of needle (*C*) and can be seen protruding from (*E*) and attached to (*C*). The needle cap (*E*) is machined from bar stock aluminum, tapped to fit threads on (*C*) and the edges knurled as illustrated. The indwelling catheter assembly intact is shown (*H*) threaded into the open slot of a 15-gauge hypodermic needle (see cross sections *I* and *J*). A somewhat similar venipuncture needle has been reported for clinical use (2).

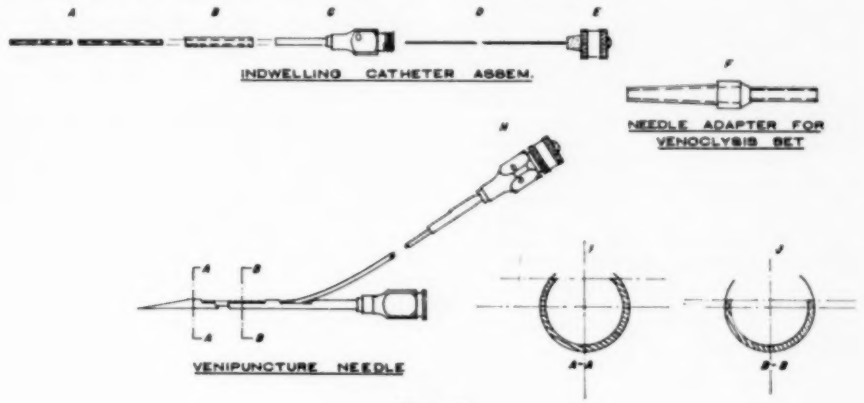

Figure 1

The stylet (*D*), rubber stopper, and cap (*E*) when in place between injection periods assure a patent capillary tube for repeated infusions. Anchoring the needle to the skin, and closure of the cannula by cap (*E*) served for a simple yet effective external protection.

Sixteen rabbits have been catheterized without and 37 with the catheter assembly described. When the assembly with the solid stylet was used, no occlusions formed within the tubing following 11 daily injections or 11 injections plus a fourteen-day recovery period.

* Received October 16, 1951, from the Division of Pharmacology and Toxicology, Atomic Energy Project, School of Medicine, University of California, Los Angeles.

† This article is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

‡ We wish to express our appreciation for the courtesies and assistance extended by Becton, Dickinson and Co., Rutherford, N. J.

[1] Supplier: Supreanant Mfg. Co., 172 Sterling St., Clinton, Mass.

REFERENCES

(1) Aurin, F. B., and Herrmann, L. G., *Am. J. Surg.*, **76**, 586(1948).

(2) Anderson, L. H., *J. Lab. Clin. Med.*, **36**, 645(1950).

Book Reviews

Textbook of Organic Chemistry. 3rd ed. By GEORGE HOLMES RICHTER. John Wiley & Sons, Inc., New York, 1952. vii + 762 pp. 15.5 x 23.5 cm. Price $6.75.

Dr. Richter believes that "the beginning textbook must be comprehensive enough to cover the broad outlines of the subject (of organic chemistry), and the treatment of the material intensive enough to provide the expected proficiency." His book represents a good attempt to satisfy the requirements, especially with regard to the breadth of its scope. All the included text material is good, but some of it may not be easily understood by some beginners.

The book is well written and the text should hold the interest of the student, especially when the author swings into such topics as octane ratings of gasoline, the newly added biosynthesis of sugars, the practical applications of organic compounds, etc. Among the newer products discussed are: silicon polymers, DDT, 2,4-D, benzedrine, chloromycetin, cortisone, vitamin E, paludrine, phenothiazine, penicillin, and thiouracil. Many medicinal compounds are mentioned without indicating synonyms or official names. Benzedrine is mentioned, but not amphetamine (*dl, d, l*) or dexedrine; paludrine, but not chloroguanide hydrochloride. Thiouracil is mentioned, but not propylthiouracil. However, this book is intended for the students of a general course in organic chemistry rather than as a coordinated text in a pharmacy curriculum. It should serve its intended purpose well, since the third edition has been brought quite up to date by the inclusion of new material not so adequately treated in the 1943 edition. For example, the synthesis of several isotopically tagged compounds is described, and the mechanism of reactions of unsaturated systems is treated theoretically. In addition, several new types of reactions have been introduced, and optical nomenclature of sugars and amino acids is stressed.

Untoward Reactions of Cortisone and ACTH. By VINCENT J. DERBES and THOMAS E. WEISS. Charles C Thomas, Publishers, Springfield, Ill., 1951. ix + 77 pp. 14.5 x 22.5 cm. Price $2.25.

The untoward reactions that may result from poorly directed, promiscuous or prolonged use of cortisone and ACTH have been collected in this concise book. These therapeutic agents are relatively new and the periods of observation of their clinical behavior is correspondingly limited. Some of the earlier noted untoward reactions have now been utilized for beneficial therapy.

The physiologic responses initiated by the administration of cortisone and ACTH are reviewed briefly. The untoward, and in most cases undesirable, reactions that have been noted in the literature are presented under the following divisions: Glands of Internal Secretion, Electrolytes, Cardiovascular System, Infections, Musculoskeletal System, Changes in Skin, Subcutaneous Tissues and Mucous Membranes, Central Nervous System, and Gastrointestinal Tract.

Careful selection of patients and close attention to details can be expected to minimize most untoward reactions except in patients who require excessively large doses of cortisone or ACTH over prolonged periods to control disease. The methods of treatment for patients showing untoward reactions to these drugs are cited. A bibliography of 96 references and a subject index are included.

BOOKS RECEIVED

Chemical Calculations. By SIDNEY W. BENSON. John Wiley & Sons, Inc., New York, 1952. xi + 217 pp. 15 x 23 cm. Price $2.95.

Inorganic Pharmaceutical Chemistry. 5th ed. By CHARLES H. ROGERS, TAITO O. SOINE, and CHARLES O. WILSON. Lea & Febiger, Philadelphia, 1952. 850 pp. Illustrated. 16.5 x 24 cm. Price $10.

Vinyl and Related Polymers. By CALVIN E. SCHILDKNECHT. John Wiley & Sons, Inc., New York, 1952. xi + 723 pp. Illustrated. 15.5 x 23.5 cm. Price $12.50.

The British Pharmaceutical Codex 1949, Supplement 1952. Published by direction of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, 1952. xii + 148 pp. 14.5 x 23 cm. Price 25*s*.

The Mitotic Cycle. By ARTHUR HUGHES. Academic Press, Inc., New York, 1952. viii + 232 pp. Illustrated. 16 x 25.5 cm. Price $6.

Scientific and Technical Abbreviations, Signs and Symbols. 2nd ed. By O. T. ZIMMERMAN and IRVIN LAVINE. Industrial Research Service, Dover, N. H., 1949. xiv + 541 pp. 21.5 x 15 cm. Price in U.S., $8; Foreign, $9.

The Physiological Foundation of Dental Practice. By L. L. LANGLEY and E. CHERASKIN. C. V. Mosby Company, St. Louis, Mo., 1951. 511 pp. Illustrated. 18 x 25.5 cm. Price $8.25.

Folic Acid and Its Pharmaceutical Application. Calco Technical Bulletin No. 729. American Cyanamid Company, Calco Chemical Division, Bound Brook, N. J., 1951. 28 pp. 21.8 x 28 cm.

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